



Hyperspectral User Manual



IMPORTANT: PLEASE READ CAREFULLY



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If any component of the Equipment does not function properly during the Warranty Period due to defects in material or workmanship, CytoViva will, at its option, either repair or replace the component without charge, subject to the conditions and limitations stated herein. Such repair service will include all labor as well as any necessary adjustments and/or replacement parts. If replacement components are used in making repairs, these components may be remanufactured, or may contain remanufactured materials. Repair or replacement without charge is CytoViva's only obligation under this warranty. This warranty is NOT transferable from the original purchaser of the Equipment.

Limitations

Other components of the product package, specifically including, but are not limited to, the light source(s), light source power transformer(s) and cord(s), liquid light guide(s), optical filters, spectrophotometer, camera(s), software, microscope part(s), and motorized stage are warranted based on the individual original manufacturer's warranties and policies. There is no warranty whatsoever on the contrast filters, bulbs or the coil (on the motorized stage) purchased as part of the Equipment.

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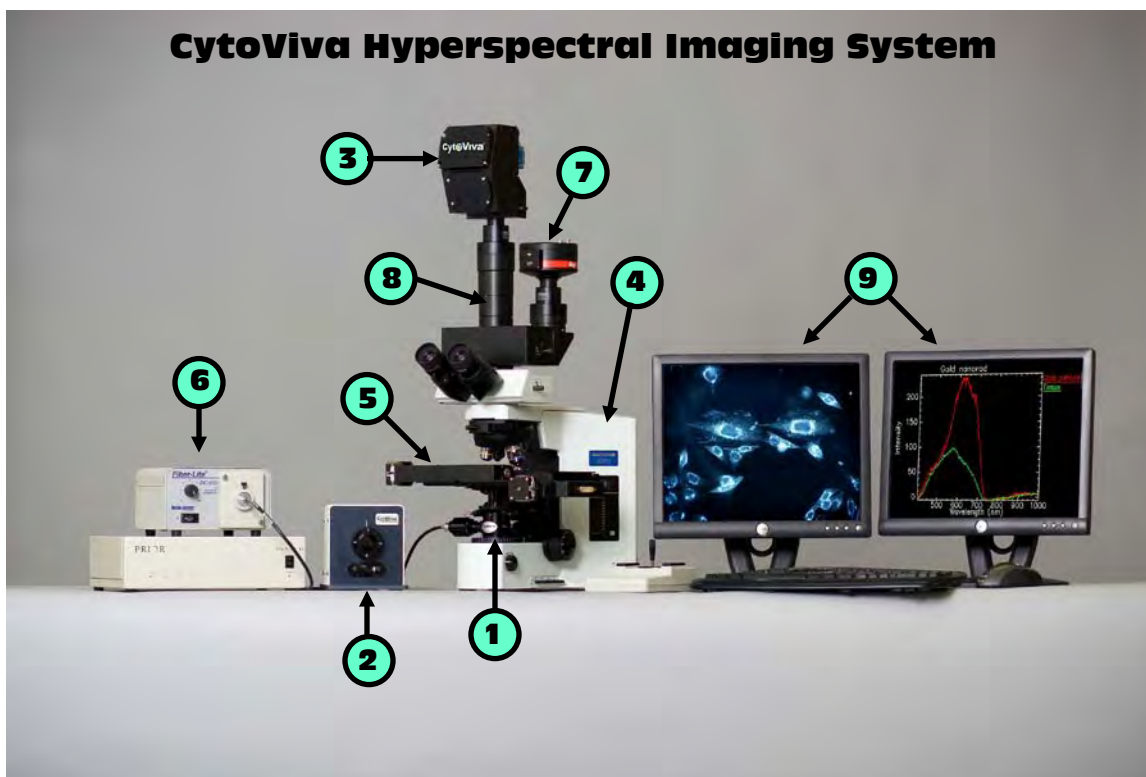
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Chapter 1: CytoViva Hyperspectral Imaging System Installation



- ① CytoViva High Resolution Adapter
- ② CytoViva Dual Mode Fluorescence (DMF) Module
- ③ VNIR Hyperspectral Imager
- ④ Optical Microscope
- ⑤ Motorized Stage
- ⑥ 150W Halogen Light Source
- ⑦ Optical Camera
- ⑧ Dual Port
- ⑨ Computer

Chapter 2: Quick Start Guide

Part 1: Starting CytoViva ENVI Software

1. The CytoViva HSI microscopy system is started by first running ENVI.
 - a. Double Click the ENVI icon that appears on the desktop to start ENVI
 - b. The main ENVI toolbar will open and all of the operations for files, image display and data analysis are contained in the drop down menus of the tool bar
2. In the menu bar find the CytoViva tab located near the right of the tool bar and begin an imaging session by selecting the menu and clicking on the contents

****Be sure that the stage controller has been turned on and that it is connected to the computer before proceeding further. If the stage is not turned on at this time, an error message will be generated and stage movement may occur after the controller is turned on****

3. Enter the COM port number (see Figure 1) which has been assigned to the automated stage by your computer (usually 3).
 - a. Click OK.

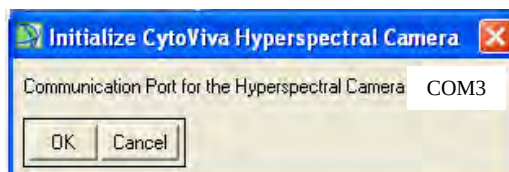


Figure 1. COM Port Window

4. After a few seconds the CytoViva Hyperspectral Camera Settings window appears (see Figure 2). The dialogs in this box are used to set up the size and other attributes of the HSI image that will be acquired.

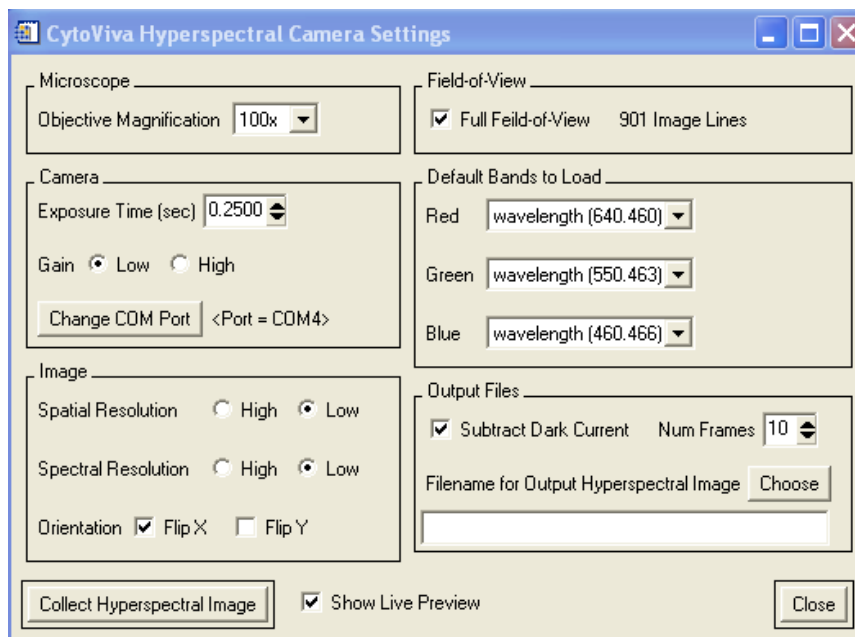


Figure 2. The control and image scanning control window

Part 2: Operating CytoViva ENVI Software

The Camera Settings dialog allows the HSI system to recognize your microscope configuration. The following controls appear in the dialog box:

Microscope:

Objective Magnification: This is set to the magnification of the microscope objective that will be used to collect the image (example 10X). The setting should always match the objective, otherwise images will look stretched or compressed from top to bottom.

Camera:

Exposure Time: Enter a camera exposure time. This value is the time that the camera will be exposed to the spectral image on each line of the HSI scan. For bright fields of view, exposures between 0.1 and 0.5 are appropriate. Longer exposures will be needed for low light such as obtained from fluorescent samples (0.5 - ∞). The time needed to complete the HSI image collection is proportional to the exposure time that you enter. Either use the scroll bar to change the value in 1 second increments, or enter the value directly in the text box, followed by Return.

Gain: The gain refers to the sensitivity setting on the camera. Usually the gain is set low. High gain can be used for low light imaging, however image quality is better if the gain is kept low and the camera exposure is lengthened. If photobleaching is a problem, use of high gain will allow a shorter camera exposure, and thus a faster scan that limits the amount of bleaching.

COM Port: You will not need to change the COM port so do not use this feature.

Image:

Spatial Resolution: Either "high" or "low" spatial resolution can be selected for the HSI image data. Low resolution is recommended since this mode results in smaller file sizes and increased image brightness. Usually brightness is preferred over resolution to get the most information from an HSI image. High resolution effectively doubles the spatial resolution, however the HSI file sizes are also significantly increased. The default resolution is "low".

Spectral Resolution: we recommend setting it to low. The spectral resolution when high is selected is 1.25nm and when low is selected it is 2.50nm.

Orientation: Make sure only the FlipX box is checked so that the image will be displayed as a direct view instead of a mirror image.

Field of View:

The number of scan lines that are needed to achieve a square image scan is calculated by ENVI. You may want to record from a smaller area of the field of view. To save time and reduce the size of the image file, the number of lines can be reduced. Change the number of lines by un-checking the box, enter a new number and press return. Remember that more lines will require a longer scan. If the area of interest is located only in the center of the field of view near the intersection of the eye piece cross hairs, 200-300 lines will be sufficient.

Default Bands to Load:

The spectral image bands that will be used to represent red, green and blue are selected using the drop down lists for each color. The default bands will give a true color representation of the data. When the HSI data does not contain intensities at a default wavelengths, other image bands can be selected to represent one of the colors. To choose a new band to represent a color, press the down button. A list of the HSI bands and associated wavelengths for each band appears. Scroll through the list and select a band. The dialog jumps automatically from red, to green, and to blue as new selections are made.

Output Files:

Dark values can automatically be subtracted from the image data during the scan. Check the box to enable this feature. Enter the number of frames to average for subtraction. The default value of ten is recommended.

The folder and name that will be given to the HSI image is selected next. SelectChoose. Browse to the desired folder and enter a new file name. A new folder for the file can be created if desired. It is helpful, though not necessary, to add the extension .img to identify the file as an HSI image.

*****Important: If an old filename is used, for example by rescanning without changing the filename, the old file will be overwritten by the next scan and the old data will be lost.****

Part 3: Starting the Scan

1. After entering the camera settings, the HSI scan is started by pressing Collect Hyperspectral Image at the lower left
2. A Live Preview of the spectral image can be included before the scan to help in the selection of the camera exposure time. This information is used to:
 - a. confirm the placement of objects in the field of the scan
 - b. to aid in adjusting focus
 - c. to confirm that camera exposure is appropriate.
3. To see the Preview dialog, check Show Live Preview before starting the scan
4. Show Live Preview was selected, the HSI Microscopy Preview screen appears (see Figure 3). This screen is used by first putting the cursor inside its borders. If the cursor is outside the window, some information will not be displayed. The upper panel of Preview shows a histogram containing the number of image pixels that have values between zero and the maximum value. Under the histogram, the panel displays a live spectral image that is being captured by the camera. In this image, the horizontal axis represents the X axis of the HSI image that will be recorded. The vertical axis represents the wavelengths of the spectrum captured in the HSI image. In the image below, the vertical streaks are spectra from objects that are in the view of the HSI camera. This image shows that two bright objects are in the view. Overlaid onto the spectral image is an intensity plot. Placing the cursor at any place on the image corresponding to a spectral wavelength causes the intensities of all the points across the image, at that wavelength, to be plotted.

Placement of objects: The features on the specimen that will be scanned are first moved into the field of view of the eyepiece and centered at the intersection of the cross hairs using the stage joystick. With the specimen positioned thus, the stage can be moved forward and backward small amounts to center the desired area on the crosshairs.

****Note any places where the features are especially bright and move these so that they intersect the horizontal crosshair. The spectra of these same objects will appear in the Preview screen****

**** Note that the live frames are flipped (reversed) about the X axis. The spectral images below are from two bright objects that appeared directly above the horizontal crosshair****

5. Focus: Typically if the objects are in focus in the eyepiece of the microscope then they are in focus to the HSI camera. Often the thickness of the specimen makes it difficult to properly adjust for best focus. This can be done more easily with the Preview image. If focus is poor, the spectra will appear blurred (see Figure 3). When focused correctly, the spectra become sharp (see Figure 4). Focusing with the Preview window insures that the HSI image will be sharp.

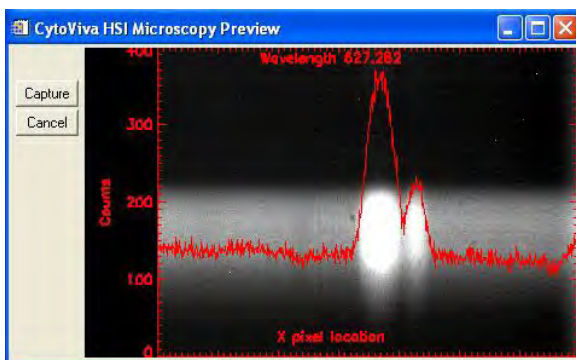


Figure 3. Frame out of focus

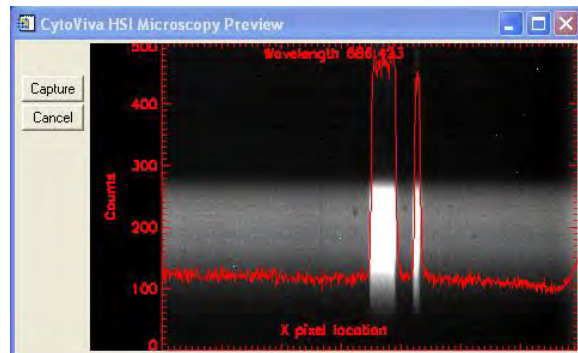


Figure 4. Frame in Focus

6. Camera exposure: The intensity plot can be used to adjust the camera exposure. Begin by moving the brightest image features to the crosshair. If the exposure is too long, the intensities will be seen to "flat top" on the plot (see Figure 5). If this should occur, the camera is overexposed to these areas and data will be missing in the HSI image. If the exposure is too short, the spectral data will be noisy.

****The best exposure will be obtained when the intensity of bright features is near 3000. Intensities of a count under 200 don't yield sufficient spectra data. Intensities will clip around 4000. The intensity values appear on the plot ordinate****

The plot scale will adjust to more and less intense parts of the spectral image as you move the cursor. The most intense readings will occur at the spectral peaks, and thus the cursor should be placed at the brightest portion of the spectra to insure that the HSI image will not "flat top" any of the recorded spectra. Usually the spectral peak is near the center wavelength, so the cursor would be placed halfway between the lower and upper ends of the spectrum.

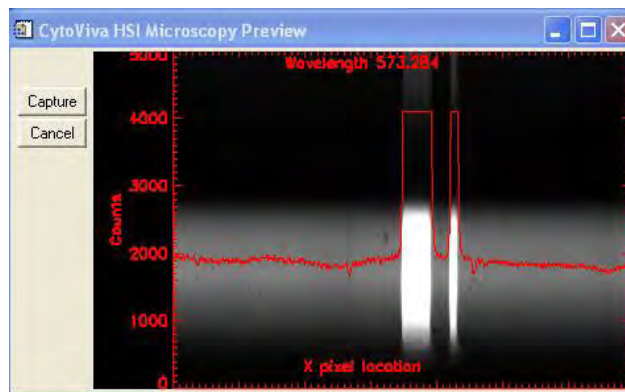


Figure 5. Intensity Plot showing "flat top"

7. Make changes to the camera exposure by canceling the Preview window and returning to the Camera Settings dialog
8. After making a change, go back to the Live Preview by pressing Collect Hyperspectral Image and review the new intensities
9. When the Preview shows good data, press Capture to start the scan.
10. If dark subtraction was selected, the following message appears (see Figure 6). **Do not press OK yet**

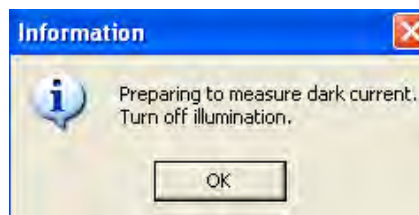


Figure 6. Dark Subtraction Window

11. At this point, divert the light path from the HSI camera to the microscope eyepiece by pushing the slide bar in all the way on the trinocular head
12. Then press OK. Do not turn off or change the brightness of the illuminator. A recording of the dark image will be made from the requested number of dark frames. The dark image that will be used is the average of the recorded dark frames.

13. Once the dark currents are captured the following message to begin the scan appears next (see figure 7). **Do not click Yes or No yet**

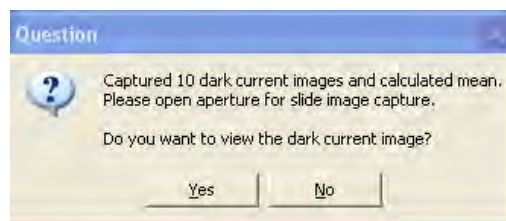


Figure 7. Dark Current Message Window

14. At this point divert the light path from the eye pieces to the HSI camera by pulling the slide bar all the way out on the trinocular head. Select No. It is not necessary to view the dark image.
15. The HSI scan will begin and the acquisition progress bar appears (see Figure 8). The stage moves to the beginning of the scanning area and then the individual lines of the HSI image are acquired, until the stage has moved to the end of the scan area. During this process, the percentage of completion of the scan is reported on the progress bar. Ignore the percent value in the lower right corner. After the scan finishes, the HSI image automatically opens in ENVI.

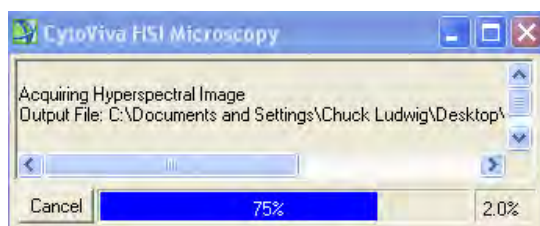


Figure 8. CytoViva HSI Acquisition Window

**** Important: if an image was rescanned using the same filename as a previous scan, the old data is lost. However, the display still contains the old images. If this happens, remove the old data from the Available bands list by selecting close all files and open the newly scanned image into the List. ****

16. To remove the old data, highlight the old data in the List.
17. Press File in the main image and click on Close Selected Files. Click through the display warning and the old data will be removed
18. Open the newly scanned File to place it in the List and obtain the new image. See Open an Existing File below for more details.

Part 4: Displaying HSI Images

The HSI image display includes three windows:

Image: This window is sized to include a user selected area of the full HSI image. The default is a square window of 400 x 400 pixels. The image can be resized by pulling at the lower right corner with the cursor.

Scroll: This window contains the full HSI image. The size and position of the Image window is marked with a red square. The red square can be moved to enter different parts of the full HSI image into the Image window.

Zoom: A sub region of the Image window appears in the Zoom window. The red square marks the shape and the position of the zoom area in the Image window. This window can also be resized and moved within the Image window. The zoom scale can be increased (+) or decreased (-) at the lower left corner of the window.

Here is the ENVI display of an HSI image of gold nanoparticles, using the color default (see Figure 9).

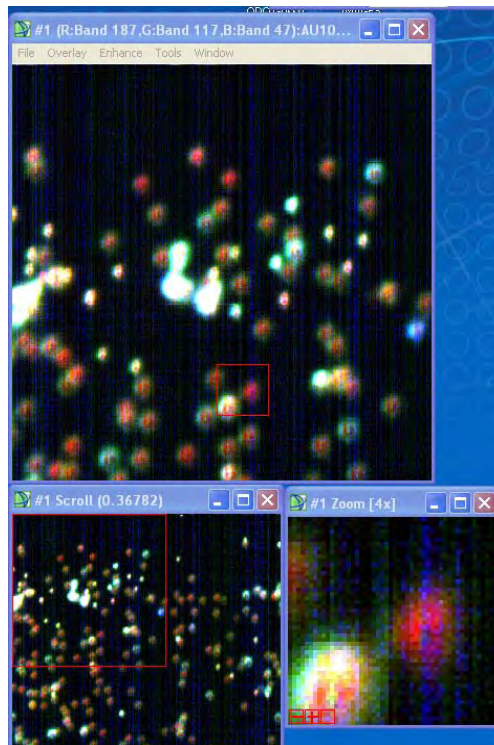


Figure 9. Color image set after scan includes Image, Scroll and Zoom displays

Part 5: Opening an Existing File

1. Using the Main Menu bar, previously recorded HSI images can be opened from the File/Open dialog. The HSI image is automatically loaded into three displays: Image, Scroll and Zoom windows.
2. Upon opening an image file, the file name appears in the Available Bands List (see Figure 10)
3. Under the name, the individual HSI image bands are listed, showing the associated wavelengths. Individual bands can be selected and displayed.

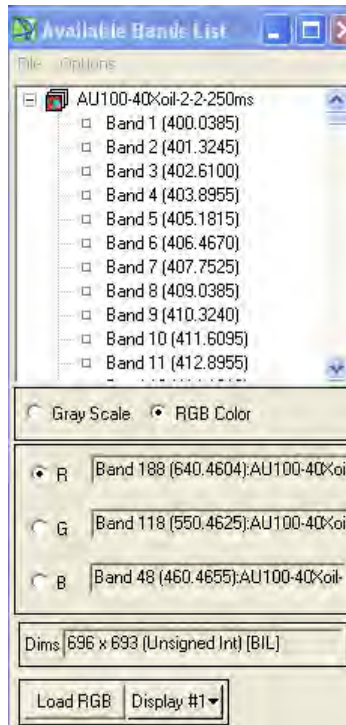


Figure 10. Available Bands List

Part 6: Selecting Display Bands

Images opened in ENVI can have different display properties. The images can be display in either Gray Scale or Color format. Color is the default format.

1. When the image is first loaded, the ENVI automatically picks which image bands to use for red, green and blue in the color display. The default bands give a true color rendering of the image. To be sure that true color is used, right click next to the file name at the top of the available bands list and select "Load True Color to Current". The newly displayed HSI image will revert to the default bands if they are not already being used (see Figure 11)
2. Other bands can be selected for red, green and blue. One reason for selecting new bands is if the HSI image is known to contain no intensity at one or more of the default bands. Changing to a different combination of bands will result in false color rendering, however it may be desirable to do so in order to create image contrast based on the features of the recorded spectrum.
3. Choose new bands by clicking to the left of red, green or blue
4. Then scroll to the desired band in the window above and click on it. After changing the image band associated with one of the colors, ENVI automatically moves to the next color
5. When you are done selecting the bands, press the Load RGB button (see Figure 12)

****When Gray Scale is selected, only one band is used to display the image****

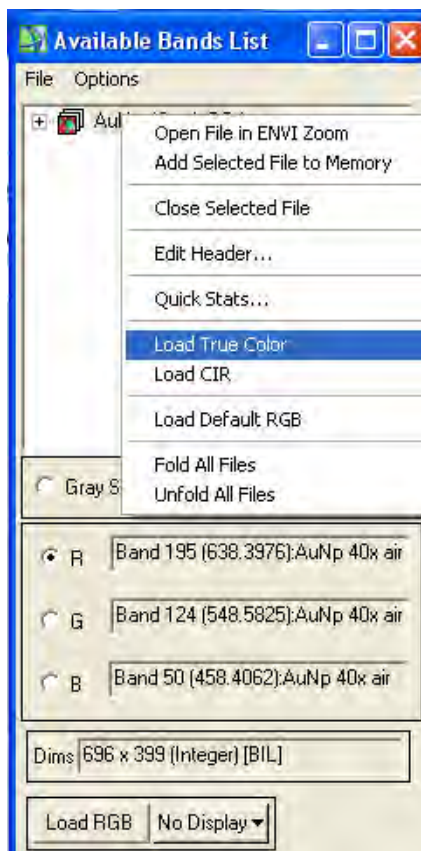


Figure 11. Load true color menu bar

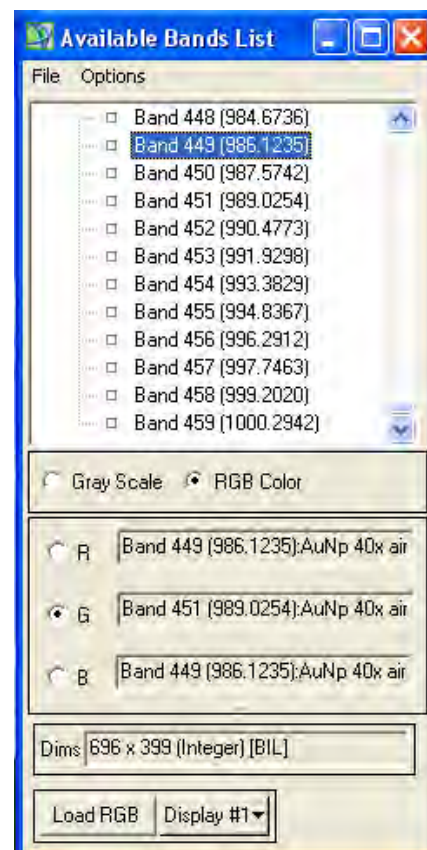


Figure 12. Automatic RGB bands

Part 7: Opening New Displays

1. Additional display windows can be opened using the No Display button on the Available Bands List (see Figure 13)
 - a. Select New Display, a blank display window appears (see Figure 14)
 - b. Open a new HSI image file
 - c. Press Load to update the blank display with the new image

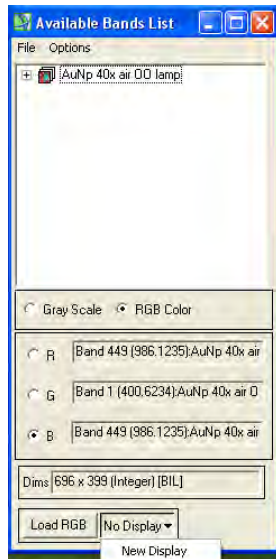


Figure 13. Available Bands List Window

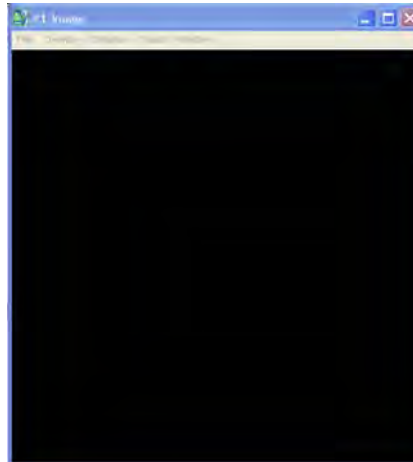


Figure 14. New Blank Window

2. Multiple HSI images that have already been opened, and appear in the Available Bands List, can be displayed in separate windows
 - a. To open multiple images from the Available Bands List (see Figure 15)
 - b. Right click on the desired image
 - c. Load True Color to <new>, will load the image into a new window
 - d. Load True Color to <current>, will load the image over the current image
 - e. Load Default RGB to <new>, will reset RGB values and open a new image window

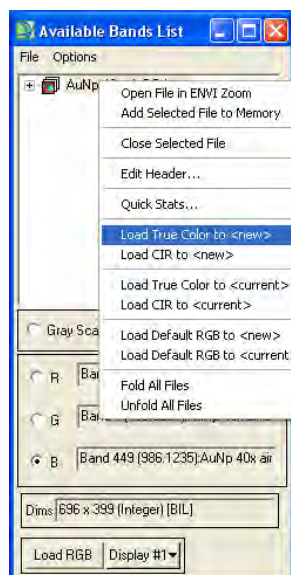


Figure 15. Imaging Open Options

Part 8: Image Enhancements

The intensity scale of the image can be stretched or compressed by using several built-in options in the Enhance drop down menu on the Image window.

Stretch Option:

1. First select the window containing the HSI image. Select the Enhance menu. A drop down list shows the built in options (see Figure 16).

****Note: stretch options can be selected for any display windows: image, zoom or scroll****

2. The stretch option will be applied to the selected window, and also to the other windows. However, only the selected window will fully adhere to the rules for the stretch option.

Linear: Causes the full range of the display to span between the lowest and highest intensity values of the image. This type of image shows the full intensity range without clipping.

Linear 0-255: Only displays values between 0 and 255. This display is used more for 8 bit images but it can also be used for faint 16 bit images to see fainter objects.

Linear 2% (default): displays between 0 and the 2% point on the input histogram. This display emphasizes very faint objects.

Gaussian, Equalization and Square Root: The stretch options "Gaussian", "Equalization" and "Square Root" perform a nonlinear transformation of the input histogram and the output histogram. "Square Root" is most useful for visualizing both faint and bright objects together, and is recommended for displaying cell and tissue samples.

3. Use the stretch options to obtain the best contrast of objects against the image background. Stretching can also reduce the appearance of vertical stripes that are caused by noise in the HSI images. Going clockwise (see Figure 17), these four stretches show the gold nanoparticles at increasing contrast against background. The strength of the stripes also increases. The "Square Root" stretching makes both faint and bright objects more visible. **Stretching the display does not affect the image data.** "Linear 2%" and "Equalization" stretching show the faintest objects.

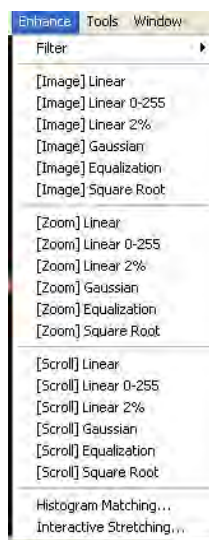


Figure 16.
Enhance drop down menu

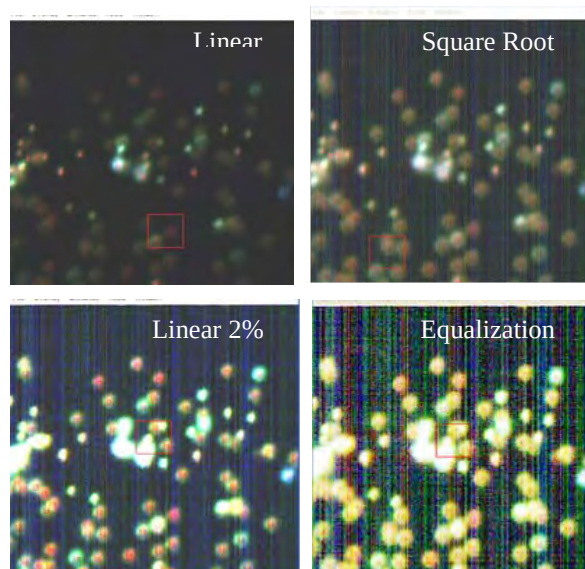


Figure 17. Examples of image with faint and bright features using different stretch options.

Interactive Stretching:

ENVI also allows the image contrast to be set interactively. This mode is selected at the bottom of the "Enhance" drop down menu: Interactive stretching.

Interactive Stretching with Input & Output Histograms:

1. The display can be adjusted by changing the relationship between the input and output (see Figure 18) histograms. This option provides greater flexibility to adjust the image display
2. The vertical dashed lines at the left and right edges of the input histogram can be moved to reset the bottom and top of the display range
3. Alternatively, these boundaries can be set by typing the new values into the stretch boxes
4. The new stretch is made effective by pressing Apply
5. Image noise is effectively removed from the display by setting the lower boundary value between 5 and 20
6. For RGB images, the process is repeated for each color by clicking the R, G, or B

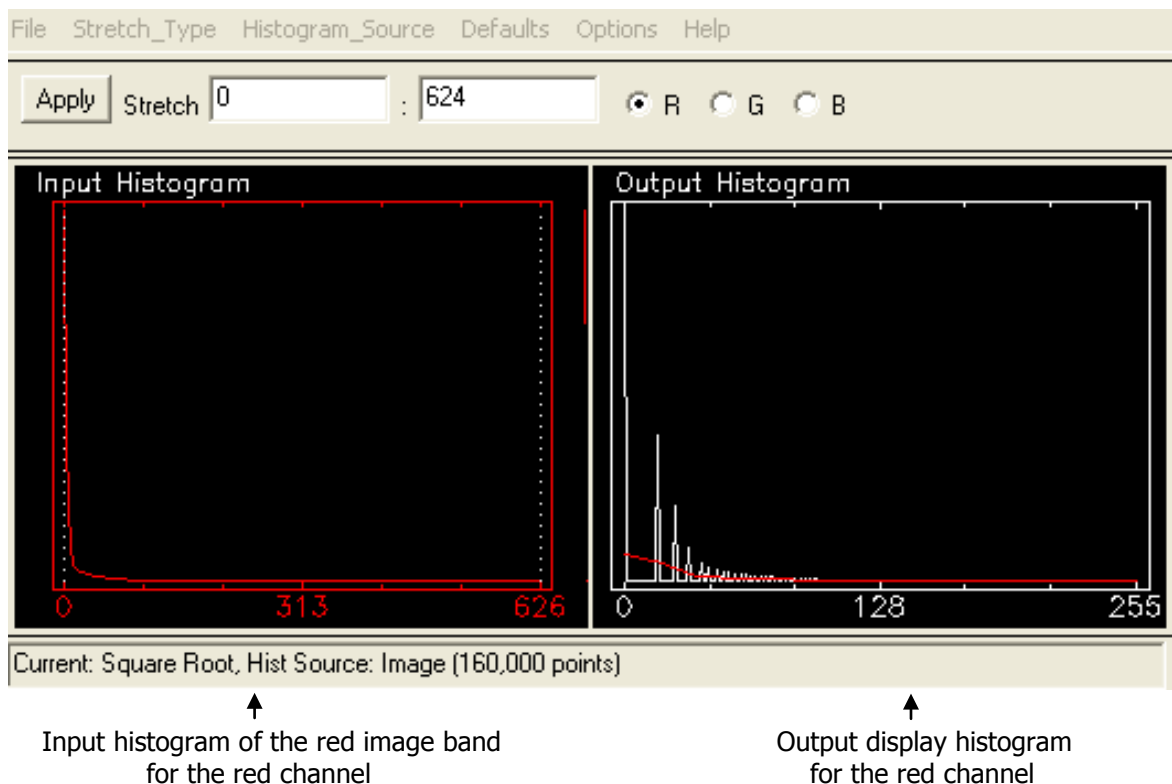


Figure 18. Input and Output Histogram

Part 9: Plotting

Intensity profiles along the X, Y and Z (spectral) axis can be plotted using the profile tool at the top of the main image window.

1. Click on Tools, then high light Profiles (see Figure 19)

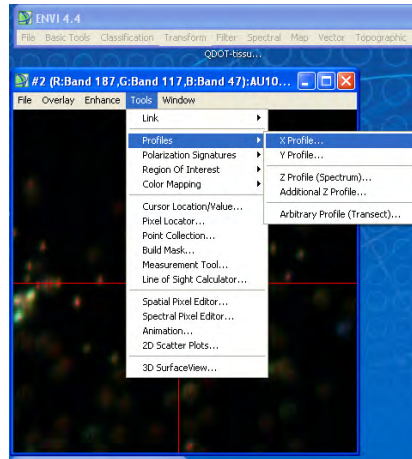


Figure 19. The Profile List is under Tools in the Image tool bar.

2. Select a profile (X, Y or Z). In this example of gold nanoparticles, the X profile was selected and then the Z profile was selected following the same procedure
 - a. When a Profile is selected, a cross hair appears on the Image and the intensity plot is displayed
 - b. The plot will contain the image intensities along the horizontal crosshair (X) or the vertical crosshair (Y), or the Spectral Profile that is contained in the HSI image data pixels at the crosshair intersection (see Figure 20)

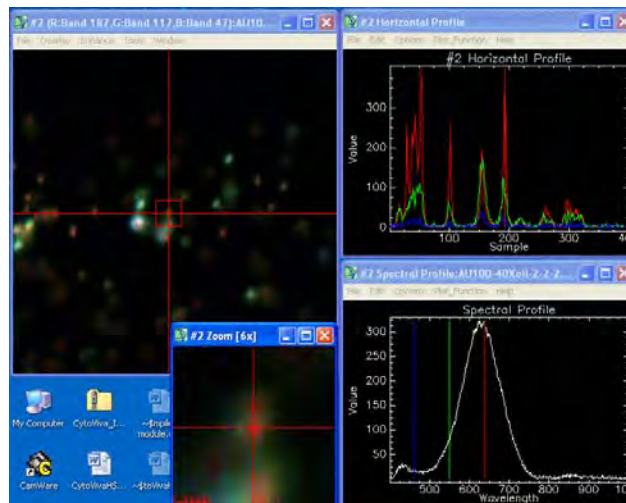


Figure 20. Plot Profiles

3. To view the Plot Key, right click in the Spectral Profile Window
 - a. Then click on Plot Key

4. To display spectra from multiple regions of the image, right click in the Spectral Profile window
 - a. Select "Collect Spectra". A new curve is added to the plot each time a new area of the image is clicked. (see Figure 21)
 - b. You can go back to the Zoom Window and select pixels to collect spectra from
 - c. In the Zoom window, clicking (+) increases the zoom while (-) decreases it. It is easier to center the cross hair on image features using a higher zoom setting

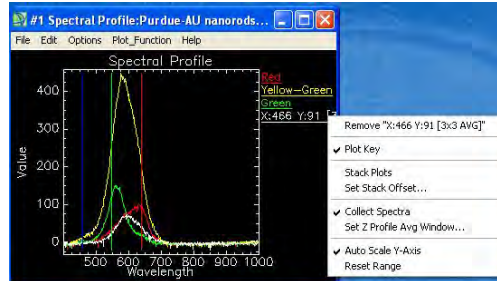


Figure 21. Window to collect spectra from multiple regions

Noise Reduction:

Noise Reduction can be applied to the Spectral Plot by averaging pixels

Averaging Pixels:

1. To reduce noise in the spectrum, right click in the Spectral profile
2. Select Z profile average window (see Figure 22)
3. By increasing the average window size to 3x3 this will significantly reduce the noise on the spectrum. Larger window sizes can be used to further reduce noise if the spectrum of interest comes from a feature that is at least this size. Excessively large window sizes will begin to include spectra from areas unrelated to the feature, changing the spectrum. Use smaller window sizes for smaller materials of interest
4. The Z Profile is useful for quickly sampling the spectrum from any part of the image



Figure 22. Z Profile Average

5. The spectral profile (see Figure 23) is marked with red, green and blue lines that show the wavelengths of the RGB display bands. The HSI band and wavelength are shown below the plot when the user clicks on the line. The default bands are "true color" wavelengths: red = 640nm, green = 550 nm and blue = 460 nm. Images will appear with normal colors using the default setting

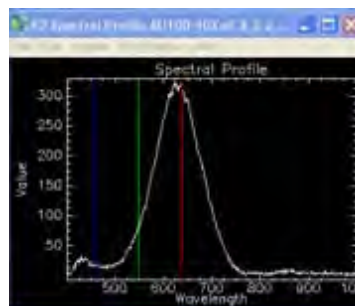


Figure 23. Spectral Profile, wavelength display

Part 10: Working with Regions of Interest (ROI)

The Region of Interest (ROI) tool is used to group pixels of the HSI image for further processing. This powerful feature allows preset shapes (rectangle, ellipse) and user defined areas (polygons, lines and pixels) to define a special region of the image.

**** ROI's allow groups of pixels to be selected for analysis and plotting. ROI's appear on the display as color overlays. ****

1. Right click in the image and select the ROI Tool
2. Expand the ROI Tools window to the right to see all ROI attributes above the box
3. The Tool Selections in this window are used to change the type of ROI, add and delete ROIs from the active list, and selecting ROIs for plotting:

ROI Type (top tool bar): Selects different drawing tools.

Window: Selects the display in which the ROI is to be drawn. Select the off button when you are only moving the ROI and not wanting to create an ROI.

New Region: Adds a new ROI to the current list. You are able to change the color by right clicking the current color and picking the new color.

Delete: Removes the ROI item in the list.

Select All: Causes all ROI's to be processed or plotted together.

Stats: Plots the spectrum of each selected ROI.

Example of creating an ROI:

1. Click on the ROI Type menu
2. Then select the polygon drawing tool
3. Select the zoom window display (see Figure 24)
4. Outline the boundary of a feature by first clicking near the feature. Then drag the cursor around the feature, clicking at points where the edge of the ROI changes direction. Return to near the first point and right click once
5. After the first click, a handle is shown that can be used to move the ROI. After the second click, the area encircled will fill in with a solid color, marking the ROI on the image. for examples created with the ellipse and the polygon drawing tools.

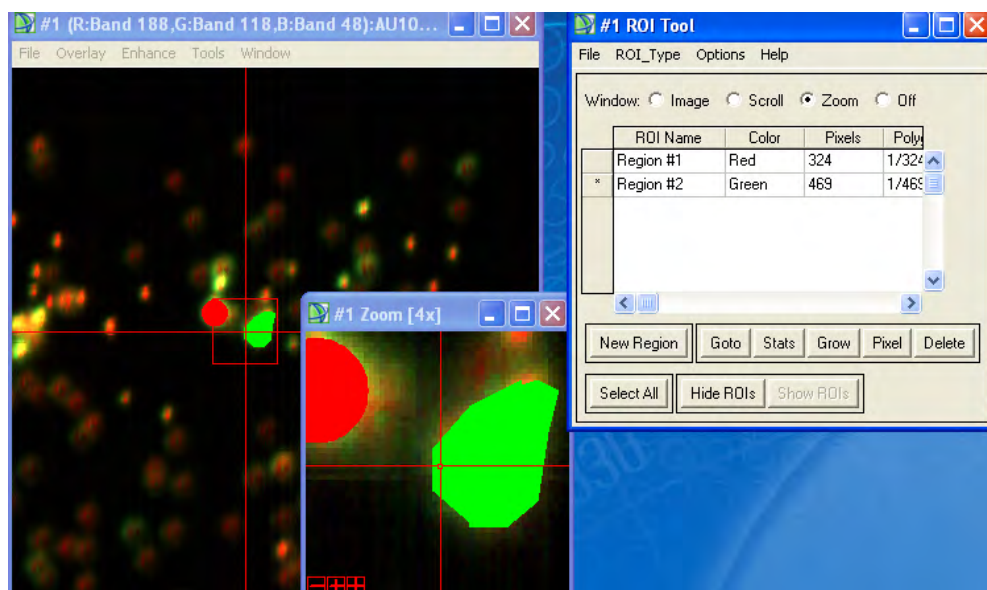


Figure 24. The ROI Tool and examples

Part 11: Plotting and labeling spectra from ROI's

Regions in the ROI list can be selected for plotting.

1. To select all regions, press "Select All". Individual ROIs are selected by clicking to the left of the name in the list.
2. Select the ROIs whose spectra are to be plotted
3. Use the Stat button to create a plot window containing the spectra for each ROI.
4. The spectral curves from the red and green ROI's above are plotted (see Figure 20, left plot)
5. A data legend was added by right clicking in the plot and selecting Plot key (see Figure 20, right plot)
6. To change the title and axis labels
 - a. Right click
 - b. Select edit
 - c. Then select Plot Parameter.
 - d. This dialog is used to change scales of the axes, the background color, style of axis markers and plot margins.
 - e. The Edit Plot Parameters dialog also allows the right margin of the plot to be made wide, so that longer names are displayed properly. Change the number in "Right Margin" to a larger value.
7. The label at the right margin shows the coordinates of the pixel used to create the spectrum. The label can be changed to a name that refers to the spectral feature
 - a. By right clicking the window
 - b. Selecting Edit
 - c. Then selecting the Edit Data Parameters dialog
 - d. Select labels appearing in the list and enter a new name in the box below
 - e. Click on Apply
 - f. In the plot at the right, the labels are changed to refer to different particles (see Figure 25)

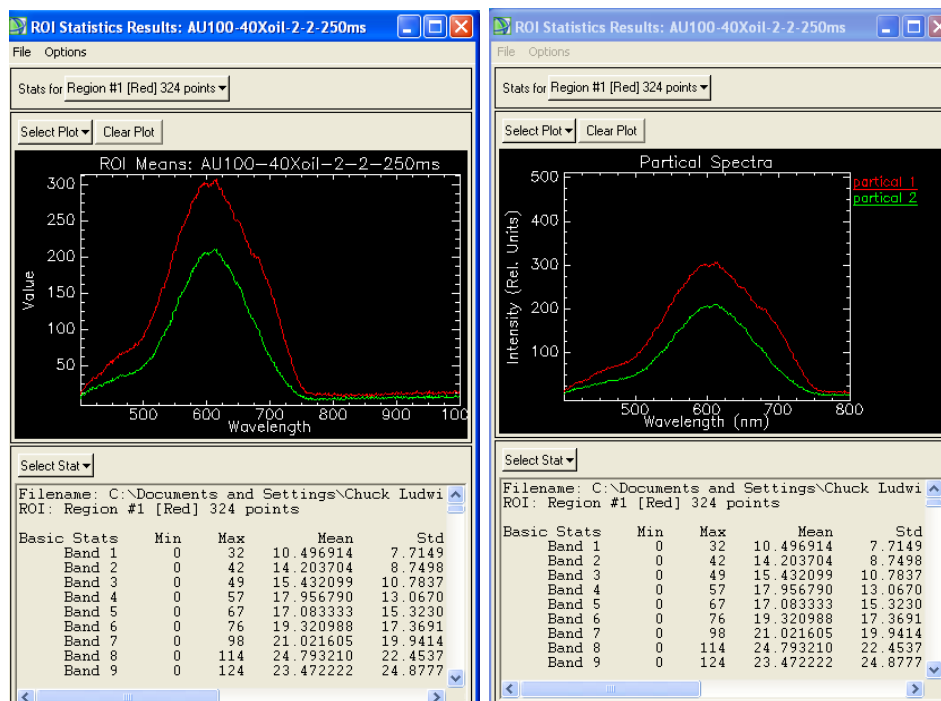


Figure 25. Spectral plots of ROIs.

Part 12: Saving Images

The hyperspectral image data is saved when the image is scanned, before an image is displayed. This is why the filename for the HSI file must be given before scanning. There is no need to save this data again. ENVI can open images from files and display them, or bring selected HSI bands into a gray scale or RGB image display. These display images can be saved in various formats. It is possible to save the main image and the zoom image.

1. In the main ENVI bar, click on File
 - a. Then select Save Image As
 - b. Then select Image File
 - c. The Output Display to Image File dialog opens (see Figure 26)
 - d. If the image is color, use 24 bit BSQ resolution. For gray scale images use 8 bit.
 - e. A portion of the image can be saved using "Spatial Subset". The number of samples (NS) is equal to the number of image columns. The number of lines (NL) is equal to the number of lines scanned, and to the number of image rows
 - f. Click the Image button to create the portion desired. You will see a red border around the image. Grab the corner and resize the border to the desired image size, then click OK.
 - g. Click OK in the Select Spatial Subset Window
 - h. The Output File Type gives a list of image types supported by ENVI. Select one of your choosing
2. To enter the filename and folder
 - a. Select Choose
 - b. Then enter the File Name
 - c. Click Open
 - d. Then click OK in the Output Display to Image File.
3. The Zoom window can be saved in exactly the same manner
 - a. Choosing Save Zoom Image
 - b. Selecting Image File

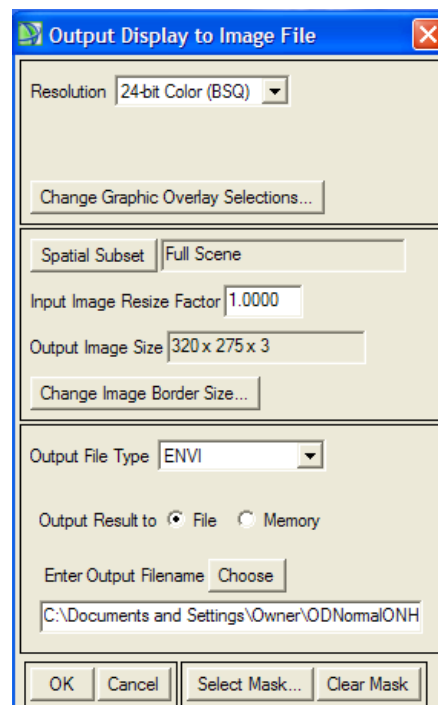


Figure 26. Output Display to Image File

Chapter 3: Extraction of Spectral Profiles

The CytoViva Hyperspectral image analysis software powered by ENVI (ITT-VIS) contains several essential features, both manual and automated, to aid the user in the quantification and identification of materials by spectral data analysis.

Part 1: Opening Test Image

In this example, we illustrate how you can extract spectra from gold nanoparticles that appear in different colors in the RGB display.

1. Open the ENVI-IDL Software by double clicking on the ENVI_IDL icon on the desktop.
2. Click on File
 - a. Select open image file
 - b. Select CytoViva Test Images (These test images are preloaded onto your ENVI software)
 - c. Select AU nanorods – 40x.img
3. Load the image into a new color display (see Figure 1)
4. Set the display enhancement
 - d. Using the Image Menu click on the Enhance drop down list
 - e. Click on Linear 0-255
3. Observe that there are small particles spread over the image, and that the particles are mostly red, yellow and green. The colors viewed in the display are determined by which bands are chosen to represent red, green and blue in the available bands list. Although the particles appear to have different color properties, we need to see if the spectra of the different colored particles are similar or very different
4. In the figure above, the Zoom window area is marked by a red rectangle.



Figure 11. AU nanorods

Part 2: Acquiring and Labeling a Spectral Profile

Spectra can be plotted from image features that are selected by the cursor. This action uses the Z Profile feature.

1. Enlarge the Zoom window so that it contains many objects of different color (see Figure 2)
2. Increase the zoom factor so that individual pixels are observed by using the (-) and (+) tabs in the zoom window
3. To turn on and off the crosshairs use the button to the right of the (+) in the bottom left corner of the zoom window. The crosshair cursor appears below in the Zoom window (see Figure 2)

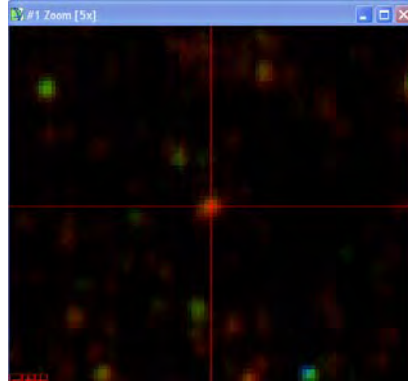


Figure 2. Zoom window with crosshair enabled and centered on particle

4. Pulling up the plot window:
 - a. Using the Image Menu Bar
 - i. Select Tools
 - ii. Select Profiles
 - iii. Then select Z Profile (Spectrum)
 - b. Or right click in the zoom window and select Z Profile (Spectrum)
5. In the Zoom window, move the cursor, now in a crosshair format, over a red object
6. Left click in the zoom window to obtain the spectra of the pixel under the cursor in the spectral profile window (see Figure 3).
 - a. The arrow keys on the keyboard will move the crosshair to the desired position
7. Then right click in the plot window and select Set Z Profile Average Window
8. Adjust the two window size boxes to 3
9. Click OK. You will see that there is a decrease in the noise of the plotted spectrum as the spectrum is now averaged over 9 pixels centered on the crosshair.

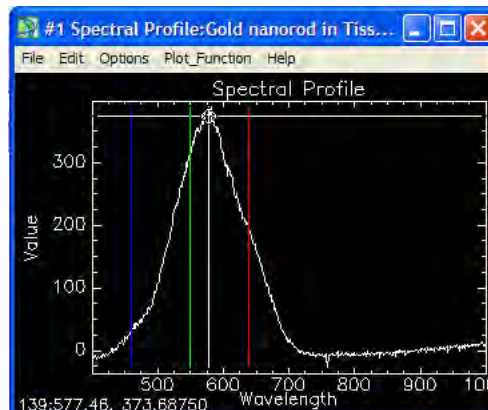


Figure 3. Plots using the Spectral Profile tool

Part 3 : Reading the Data

1. The spectrum contains data from all the image bands at a specific set of pixels chosen in an ROI or a spectral profile. The band number, wavelength, and spectral intensity can be displayed at the bottom left corner of the plot (see Figure 4)
2. Read the information from specific locations on the spectrum by left clicking and holding the button down as you move in the plot window. A line cursor follows your movement, and the band, wavelength and intensity are updated in the lower left.
3. In this example (Figure 4), the data show that the cursor is at band 139, the wavelength is 577.46nm and the intensity of the spectrum is 373.7 units.

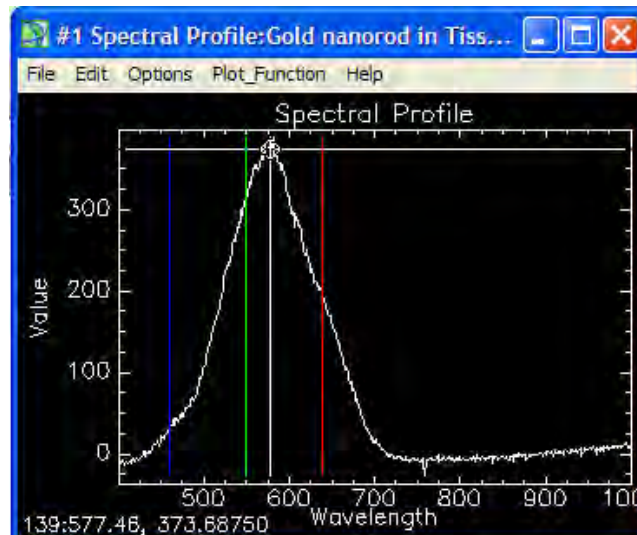


Figure 4. The Spectral Profile with vertical cursor and data readout

Part 4: Changing the Plot Key Parameters

1. In the plot window right click
2. Select Plot Key – you will see the spectral curve label name in the right margin
3. To change the Plot Key names , line color and style so that can be identified later go to the plot window and on the image menu bar
 - a. Select Edit
 - b. Then select Data Parameters
 - c. In the edit window, select the label and below enter the new name in the box below
 - d. To change the plot color and line style right click on the box and select the desired line color. For this example we named the particle spectrum "Red"
 - e. Click Apply and then close the edit window (see Figure 5)
4. You can also select Plot Parameters from the Image Bar Menu to change the X & Y Axis, Title of the Plot, and Background and Foreground color.

**** If you don't click apply the new spectral curve label will not be changed ****

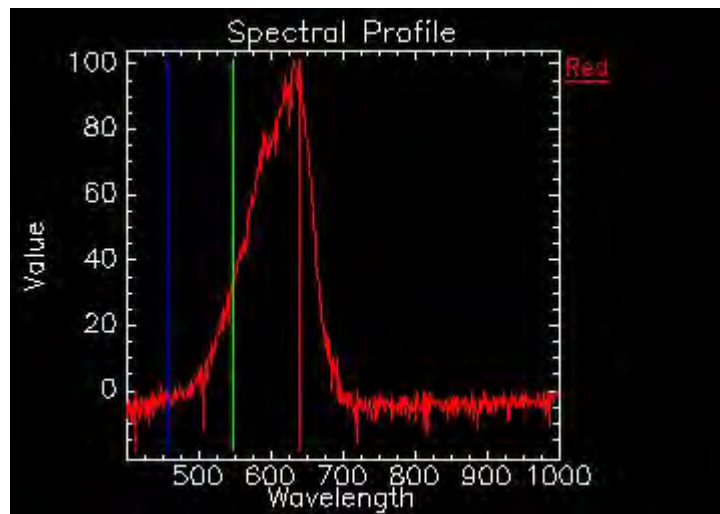


Figure 5. Labeled plot

Part 5: Collecting Spectra in the Same Field of View

1. In the Plot Window right click and select Collect Spectra. This has just collected the spectra of your red particle in the sample.

****When the Collect Spectra is selected, ENVI will collect spectra from any point clicked in all windows (Image, Scroll or Zoom Window)****

2. Now to collect spectra of another particle.
 - a. In this case we will collect the spectrum of the particle that appears yellow. Put the crosshair cursor on the desired particle by left clicking in the zoom window and moving to desired location
 - b. The new plot and its label appear together with the red spectrum and label.
3. Repeat again for a green particle.
4. You may end up with three very distinct curves (see Figure 6)
5. Finally, change the names and colors of these new spectra so they can be recognized later (Refer to Part 4: Changing the Spectral Curve Label name)

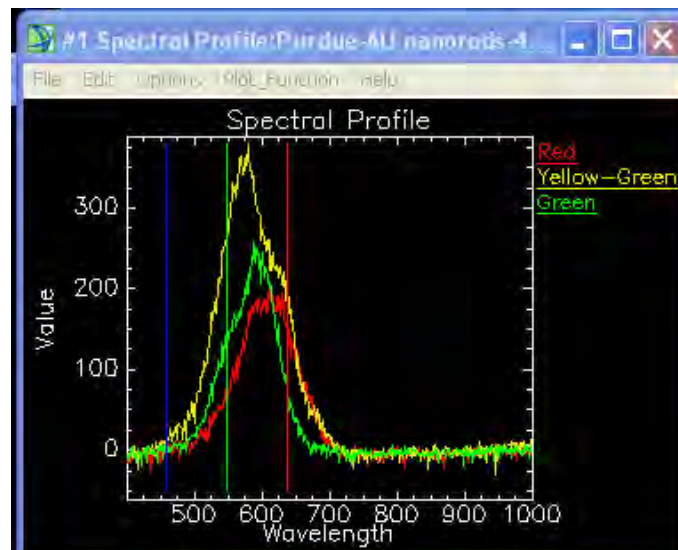


Figure 6: Spectral curves from three nanoparticles

Part 6: Collecting Spectra from Different locations in the Image

Multiple spectra can be plotted in the same window. This provides an easy way to visually compare the spectral curves of different features. Although points for spectra can be chosen from any of the display window, it is easiest to use the Zoom window, where individual pixels can be identified.

1. In the Plot Window right click and select Collect Spectra. We have already collected the spectrum of the red particle.

****When "Collect Spectra" is selected, ENVI will collect spectra from any point clicked in all windows (Image, Scroll or Zoom Window)****

2. Now collect spectra of other particles
 - a. We will collect the spectrum of the particle that appears yellow
 - b. Put the crosshair cursor on the desired particle by left clicking in the zoom window. The image moves to place the desired feature at the center of the crosshair.
 - c. The new spectrum from this location, and its label, appear together with the red spectrum in the plot.
3. Repeat again for a green particle.
4. You may end up with three very distinct curves (see Figure 7)

****Note that the color of the spectral curve is assigned by ENVI and does not reflect the color of the feature in the display****

5. After collecting spectra
 - a. Right click in the plot window
 - b. Click plot key. This will display the names of each spectra collected. The default names will be the X and Y locations of the pixel selected for the spectra collected
 - c. Then go to edit plot parameters. This will allow you to change the title of the plot, X and Y axis title.
 - d. By selecting Data parameters you can change the line color, line style and the plot key names.
 - e. By selecting editing plot parameters you can assign the spectra to different colors
 - f. Finally, change the names and colors of these new spectra so they can be recognized later.

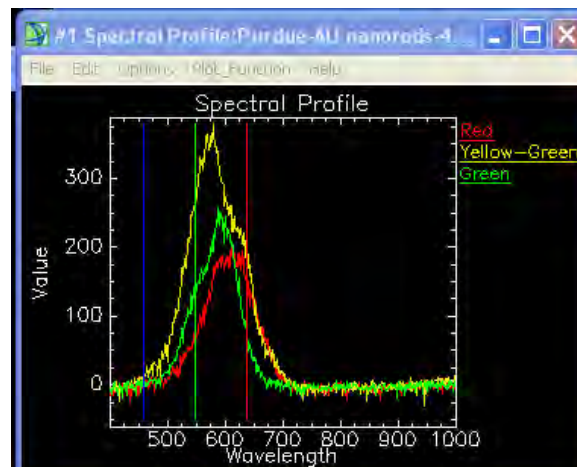


Figure 7. Spectral profiles from three image features plotted together

Part 7: Deleting Unwanted Spectra from the Plot

If you decide to remove a spectral curve, this can be done without restarting the plot.

1. In the plot window, right click on the label of the unwanted spectra collected

****Note: the first spectrum, with label at the top of the list, cannot be removed****

2. Then click on remove label (see Figure 8)
3. The selected curve will be removed.

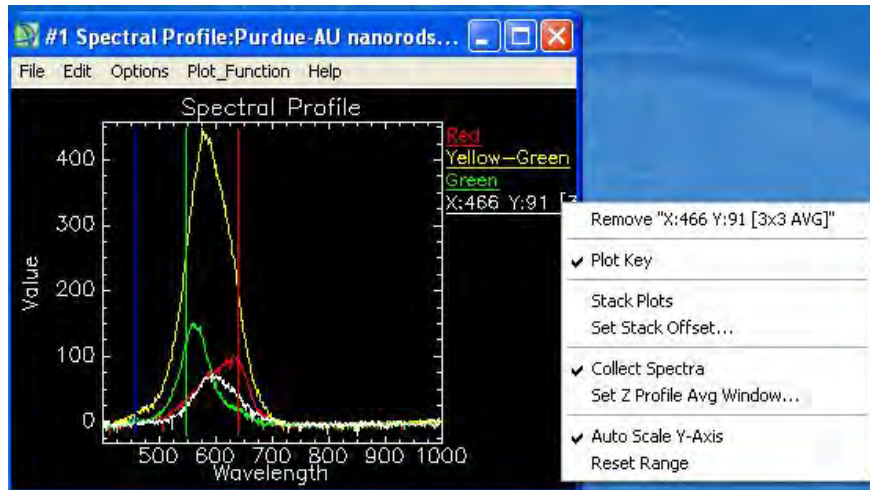


Figure 18. Option list obtained by right clicking on the plot label.

Chapter 4: Saving and Viewing Profiles in the Spectral Library

Part 1: Creating an Output Folder

When doing spectral analysis on scans you will need to create several different types of output files which could include, but are not limited to:

- Spectral library files
- Header Files
- Spectral Angle Mapper (SAM) Files

These are files of your spectral data that you will want to access later to compare against new samples. The Output folder will contain files from scanned images.

1. To create your output folder, choose a location within your images folder
2. Click on Create New Folder
3. Label the folder Output (see Figure 1)

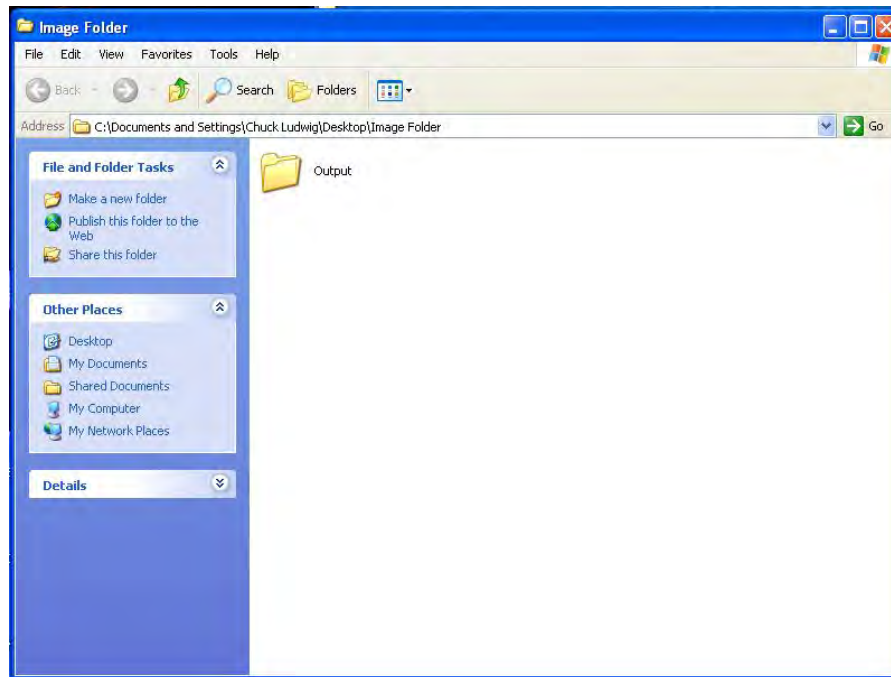


Figure 1. Output folder for saving image work in the Images Folder

Part 2: Saving and Viewing Profiles in the Spectral Library

1. After you have created your output file (refer to Part 1: Creating an Output Folder), the red, yellow, green and blue particle spectra (collected earlier) can be saved to a spectral library
2. From the spectral profile window, select File
 - a. Then select, Save Plot As
 - b. Then select Spectral Library
3. You will see the Output Plots to Spectral Library dialog box open
 - a. Select all of the plots listed in the window by clicking the Select All Items Button
 - b. Then click OK
4. You will see the Output Spectral Library dialog box open
 - a. Click the select all items button to put all spectra into the spectral library. Also individual spectra can be collected and saved into the spectral library as well
 - b. Then click OK, the Output Spectral Library window will open
5. In the Output Spectral Library window, enter the baseline intensity and the highest y axis value of the three spectral curves in the "Z Plot Range" boxes.
 - a. If curves came from imagery where the dark background had automatically been subtracted, enter -20. This value will allow the variation around zero to be included.
 - b. Do NOT click OK yet

****This information is needed for scaling spectral library curves to the size of other curves. For now, the other boxes should not be changed****

6. In the Output Spectral Library box, click the Choose button (located beside the "enter output filename") to select where the data will be saved
 - a. Go to your Output Folder and type in the chosen filename
 - b. You do not need to add an extension to the file name
 - c. Click Open
 - i. The library file and a header file are automatically saved in the Output Folder
 - ii. The library is also added to the top of the available bands list
 - d. Then click OK

Part 3: Viewing the Spectral Library File

The Spectral Library Viewer allows you to view the collected spectra individually or together.

1. In the Available Bands List Window right click on the file name of the desired spectral library file
2. Choose Spectral Library Viewer
3. In the Spectral Library Viewer select the red spectrum
4. A plot window opens with the spectral curve
5. Select the other spectra in the Viewer. They are added to the plot

****Note that curves saved in the spectral library are also referred to as "endmembers" in automated spectral classifications. The use of endmembers will be discussed next****

Chapter 5: Spectral Angle Mapper Classification

Part 1: Automated Comparison of Unknown Spectra with Spectral Libraries

The Spectral Angle Mapper Classification (SAM) is an automated procedure for determining if a known material is present in the input image, and locating which pixels contain the material. SAM accomplishes these tasks by comparing unknown spectra in hyperspectral imagery with known spectra for the material in question. The degree of match between the unknown and known spectra from each image pixel is displayed in a grayscale image known as the "Rule" image. The "Rule" image shows, on a scale of light to dark, the relative degree to which unknown spectra in each image pixel match the known spectrum. The scale is reversed from convention, so that best matches produce the darkest pixels. Since by convention, high correlations are scaled toward the bright end of the scale in image processing, the grayscale of the "Rule" image is often reversed to create this effect. Additionally, a criterion for the match between known and unknown spectra can be given. When this is requested, SAM produces a binary classification image showing locations of pixels with spectra that are within the criterion.

The SAM method works by comparing unknown spectra from each image pixel with the known spectrum (for example, in a spectral library) at each of the N wavelength bands recorded in the image. SAM first determines a vector in N dimensions that represents the distance from the origin (dark) to the light intensity recorded in each band of the unknown spectrum. The direction of this vector in N -dimensional space is used to define a unit vector representing the unknown spectrum. The same procedure is performed for the known spectrum. SAM then determines the angle between the two unit vectors. The best spectral match occurs when the angle between these vectors is the smallest.

The selection criterion that was used to make the classification image is actually a threshold for the spectral angle. Image pixels with spectral angles that are smaller than the threshold are classified as containing material belonging to the known spectrum. In plot shown below (see Figure 1), the unknown spectrum was not classified as belonging to the known spectrum since the angle turned out to be larger than the criterion allowed. The angle of each pixel is output to the "Rule" image. Because the method is based only on the direction of these vectors, and not on recorded light intensity, the SAM classification is insensitive to the illumination of the sample in the recording. Therefore, it is important that dark values are removed from the data before using SAM. Since sample illumination varies over the field of recording and between images, the SAM tool is a highly useful classification method.

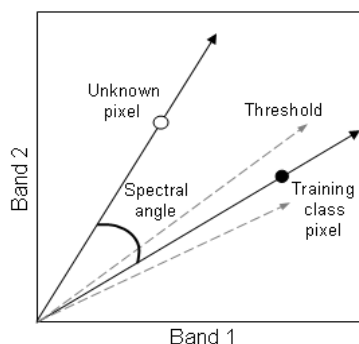


Figure 1. Threshold for classification of pixels based on spectral angle

Part 2: Using the Spectral Angle Mapper (SAM)

This example examines an image of gold nano-rods (GNR) in tissue. The object of this exercise is to automatically locate the GNR objects within the image. First, the known spectrum for GNR is acquired and saved in the spectral library. Spectra that are used as a signature for identifying a material are called endmember spectra. The SAM classification feature then is used to find the locations of objects matching the spectrum of GNR in the image.

Step 1: Acquiring the endmember spectrum

1. In the Main Menu Bar, use the Open Image File to load the input image
2. Click on the file: **Gold Nanorod in Tissue - 40Xair.img** (preloaded in your software)
 - a. To optimize the display contrast, click on Enhance in the Image Window
 - b. Then click Linear 0-255 – this will enhance the display contrast and brightness
3. Move the Zoom area over the cluster of particles near the left of the tissue section (see Figure 2).
4. Use the box in the Scroll Window to locate the area of the cluster of particles.
5. Then use the zoom box in the Image window to better locate/focus the cluster of particles so that they appear in the zoom window.

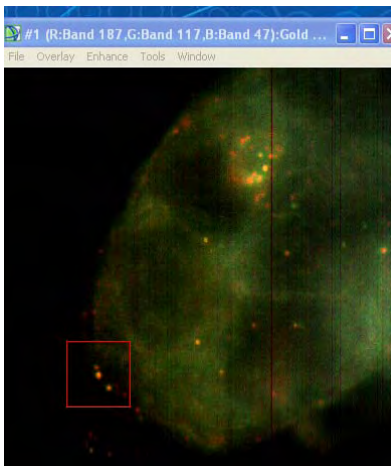


Figure 2. Gold nanorods (inside rectangle) in cell cluster

6. Using the Zoom Window, select a clear, distinct particle using the crosshair cursor (see Figure 3). We will use this particle to represent the GNR that are distributed throughout this image

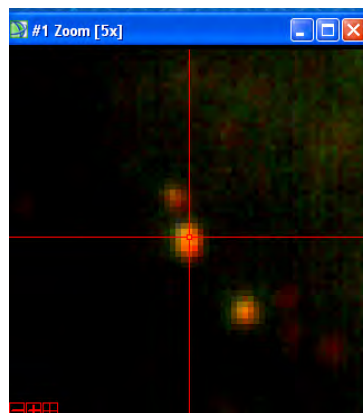


Figure 3. Crosshair centered over particle in Zoom Window

7. Now to plot the spectrum of the GNR
 - a. In the Zoom Window, right click and select "Z Profile (Spectrum)"
 - b. Before acquiring the profile, right click in the plot window and set the Z Profile Average Window to a size that fits within the borders of the particle. Here a 3x3 rectangular pixel average is selected
8. Move the crosshair cursor over the center of the particle (as in Figure 3) and left click to obtain the endmember spectrum that will be used to classify the image
9. Now give the plot key label a new name (Refer to Chapter 4, Part 4 – Changing the Plot Key Parameters) that allows you to find it in the future. Here we chose **AU NR-tissue** (see Figure 4).

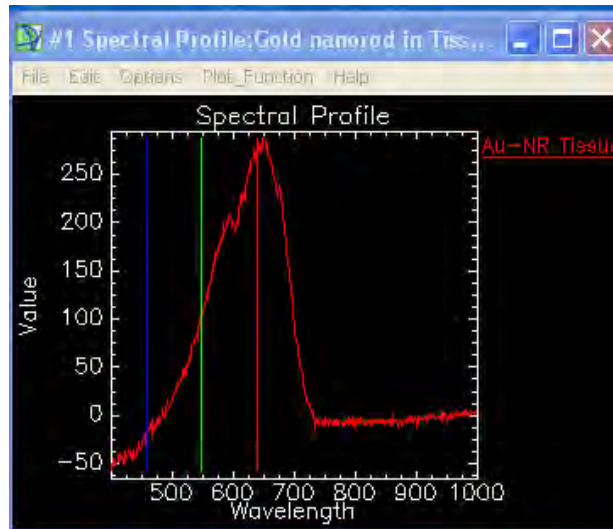


Figure 3. Spectral profile of particle with label in plot key

Step 2: Saving the endmember to the spectral library – 2 methods

Method 1: Using the Main Menu

1. From the Main Menu bar in the Image Window, select Spectral
 - a. Then select Spectral Libraries
 - b. Then select Spectral Library Builder
2. You will see the Spectral Library Builder dialog box open
 - a. Then select First Input Spectrum and click OK
3. In the plot window click and drag the label (AU NR Tissue) to the spectrum list in the Spectral Library Builder dialog (see Figure 5)

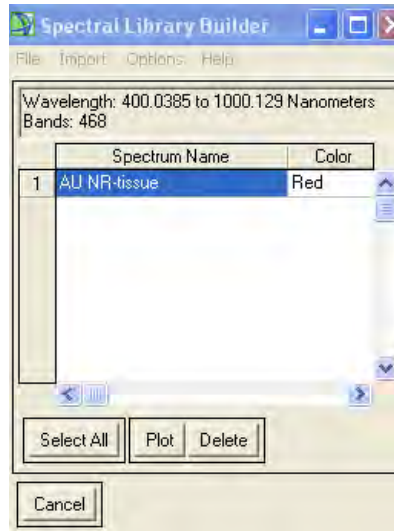


Figure 5. Spectral Library Builder dialog window

4. Click "Plot" to create a plot window that is used to accumulate the Endmember Collection Spectra (see Figure 6). In this case we have a single endmember

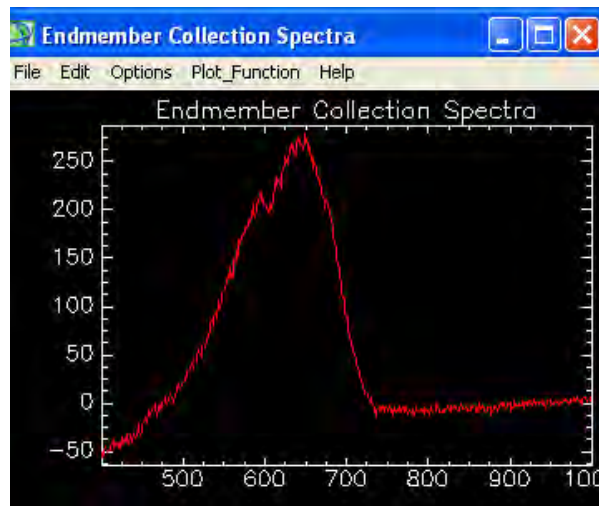


Figure 6. Plot containing the spectrum of the endmember6

Method 2: Using the Endmember Collection Spectra Window

1. In the Endmember Collection Spectra window click on File
 - a. Then select Save Plot As
 - b. Then select the Spectral Library (see Figure 7)
2. The Output Plots to Spectral Library dialogue box opens
 - a. Click Select All Items
 - b. Then click OK
3. The Output Spectral Library dialogue box opens
 - a. In this example, for Output Result, select Memory
 - b. Your spectral library is now present in the Available Bands List as data in memory

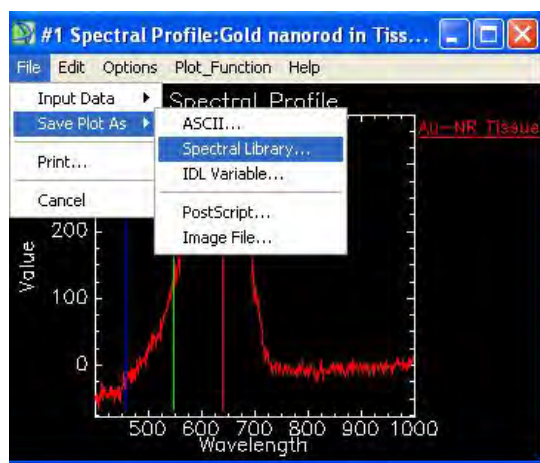


Figure 7. Saving Endmember to Spectral Library File

4. To view the endmember plot
 - a. Right click on Memory in the Available Bands list (see Figure 8)
 - b. Then select Spectral Library Viewer. This now shows the plot for the endmember(s), in this case we only have one (see Figure 9)

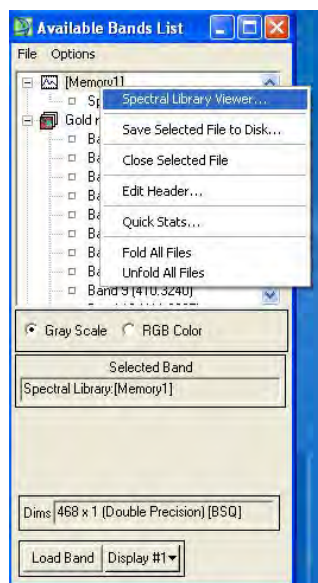


Figure 8. Using the Spectral Library Viewer to view Endmember

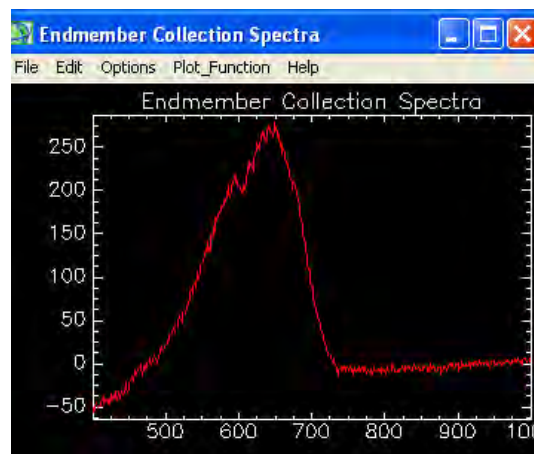


Figure 9. Plot containing the spectrum of the Endmember

Step 3: Performing the SAM Classification

We will use SAM to create a Rule image that shows how well each pixel of the input image matches the endmember spectrum. Then, we will create binary images showing which pixels exceeded the threshold for matching the endmember.

1. In the main menu bar, select Spectral
 - a. Then select Mapping Methods
 - b. Then select Spectral Angle Mapper
 - c. Then select the file in the list to use as the input image. For this exercise select **Gold nanorod in Tissue - 40Xair.img** (see Figure 10)

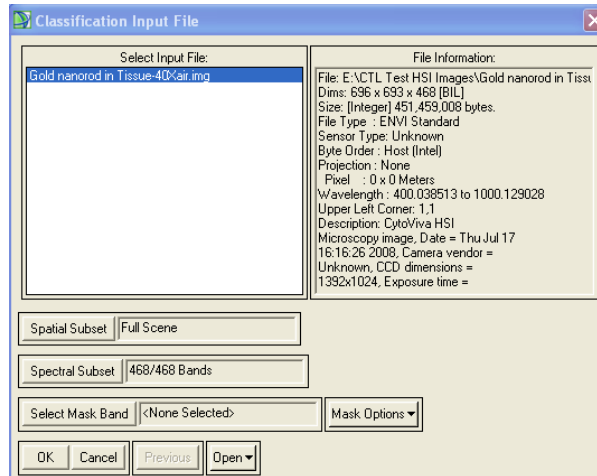


Figure 10. Selecting the input file for classification

2. For this specific example wavelengths above 800 nm provide no information for this sample image. So to exclude the bands covering wavelengths above 800 nm, use the Spectral Subset feature. SAM will create a 400-dimensional vector from 400 nm to 800 nm
3. Click on the Spectral Subset button
4. All bands are highlighted by default
5. First, remove the selection from all bands by clicking Clear
6. Then in the boxes to the left of Add Range enter 1 in the far left box and 312 in the box just to the right (this band corresponds to 800 nm) (see Figure 11)
7. Then click Add Range
8. Then click OK

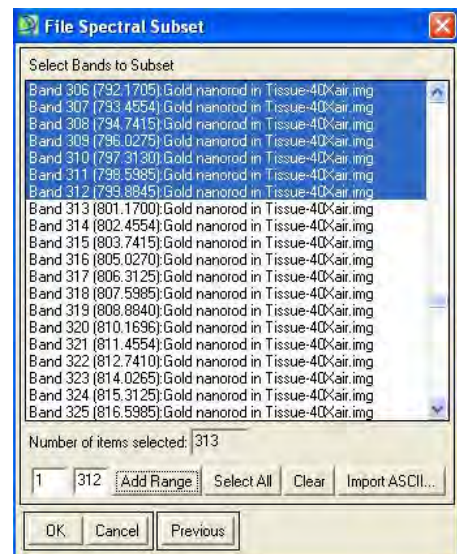


Figure 11. Select the bands by using the Add Range Boxes

9. The Spectral Subset now shows 312 out of 468 bands are selected (see Figure 12)
 - a. Click OK again

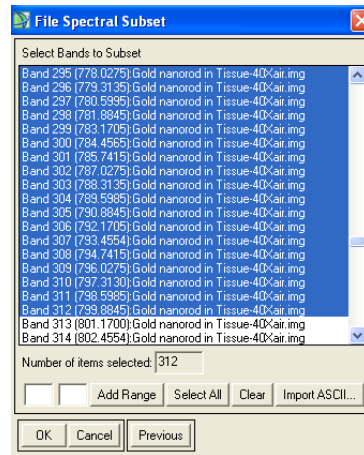


Figure 12. Selection of a subset of the spectral bands

10. You will see the Endmember Collection: SAM dialog box open
11. From the Plot Window, drag the label **AU-NR Tissue** to the Endmember Collection: SAM box. It is highlighted automatically in the list (see Figure 13)
12. Click on Apply

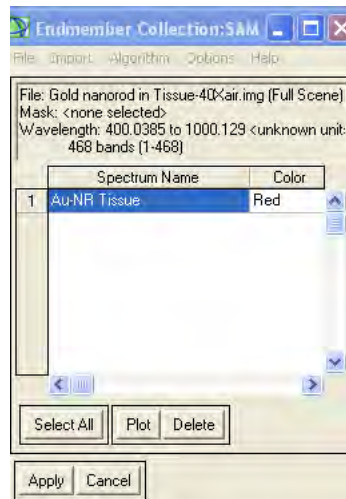


Figure 13. Endmember Collection dialog with entry from label dragged from plot.

13. You will see the Spectral Angle Mapper Parameters dialog box open
14. For Set Maximum Angle (radians), click on single value
15. Keep the Maximum Angle (radians) at 0.1. We will use this threshold first for the spectral angle
16. For Output Result, select memory. This will send the classification image to the available bands list. The Output Result image can also be saved as a file. The filename for this image should be chosen as: filename_SAM.dat
17. For Output Rule Image, select Yes
18. For Output Result, select memory. This image can also be saved in a file using the filename: filename_SAMrule.dat

19. If you want to Preview the Classified Mapped Image, click Preview (see Figure 14) from the Spectral Angle Mapper Parameter window. This will show you what pixels are going to be classified before saving.
20. After you click Preview, the preview window is added to the SAM Parameter window (see Figure 15). The overview window ONLY shows the pixels of a section of the entire image that are classified by the SAM. Unclassified portions of the sample will not be shown.

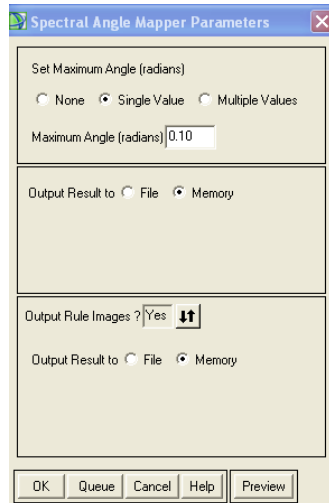


Figure 14. Window to Preview

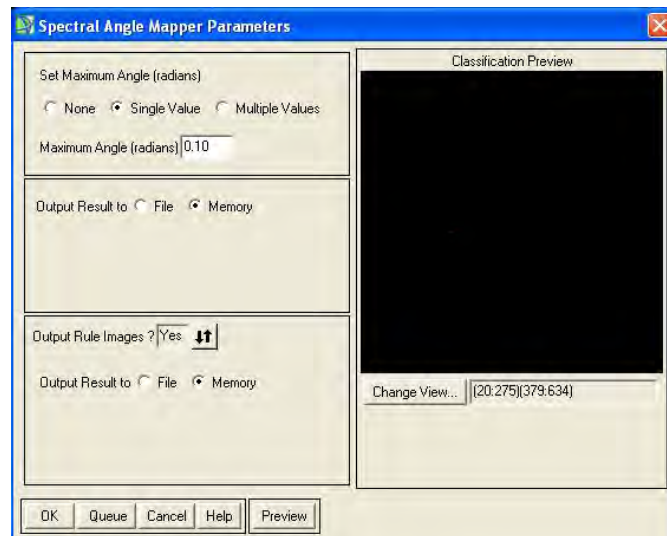


Figure 15. Spectral Angle Mapper Parameters Window with the Preview window

20. To change the preview area, click the change view button (located under the classification Preview window). The Select Spatial Subset dialogue box opens
21. Click the Image Button, the Subset by Image box opens
22. Move the red square to the desired area (see Figure 16)
23. Then Click OK in the Subset by Image Box and the Select Spatial Subset box
24. Once you have found the desired classification, click OK

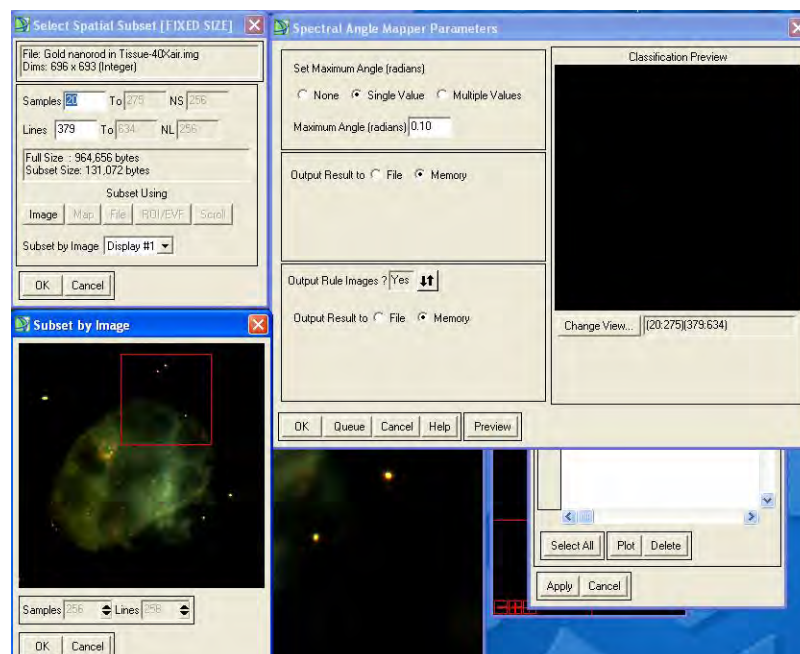


Figure 16. Spectral Angle Mapper Parameters box, Select Spatial Subset box, Subset by Image

25. The SAM files are now added to the Available bands list. Now there are two images added to the top of the available bands list
26. To load each image in a new display, right click on the Rule Image. Then select Load Band to New Display.
27. An image will open
28. Then right click on the SAM image
29. Then select Load Band to New Display
30. Another image will open
31. You should now have the original image, the Rule Image and the SAM image shown on your screen

**** For future SAM classifications, you can save Output Results and Endmember Spectra by choosing the File option rather than the Memory****

**** If you close out the ENVI software this file will be lost since it was saved to memory****

Part 3: Re-Loading Images from Available Band Lists Window

In some cases you may load one image over another by accident. This usually happens by clicking Load Band to Current Display rather than Load Band to New Display. If this happens it will load the new image over the original image. You will need to reload the old image to a new display.

1. To reload the original image (see Figure 17), scroll through the Available Bands List to find the original image. In this exercise the image name is **Gold Nanorods in Tissue**
2. Click on the filename to highlight it
3. Click Display # and select New Display
4. Click Load Band to again display the original image

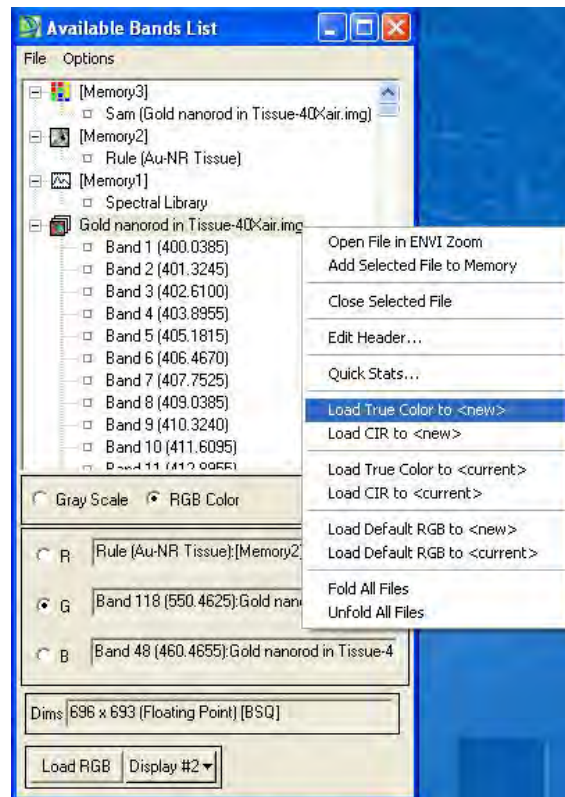


Figure 17. Available Bands List

Part 4: The Rule Image

The Rule image will show those areas that best match the Endmember Spectrum as being darker. Areas with a poorer match are lighter. For the cell cluster, the cell areas are gray and the background around cells is white. The GNR particles will appear darker than the tissue. The area around particles will also be darker, indicating that a zone around the small particles also contains the signature for that particle. These zones are produced by light scattering in the medium around the particle. The cell is gray only because it scatters more light than the background and hence its spectral curve has a greater amplitude, even though it does not match the signature of GNR. Figure 18 shows the rule image obtained by SAM.



Figure 18. SAM rule image from gold nanoparticles in cell.

Part 5: The Classification (SAM) Image:

The classification image shows the location of pixels whose spectra match that of the endmember. You will see that using a value of 0.1 for the maximum angle causes only pixels in the selected particle to be classified as a match for the endmember spectrum. The zoom window rectangle surrounds this particle, as shown in Figure 19. In the zoom window, directly below the main image you can see that three pixels were classified. The rest of the pixels within the particle contained sufficiently different spectra, as did those of every other particle, and they were rejected as matches for the endmember. This outcome suggests that the spectral angle chosen for the classifier is too small.

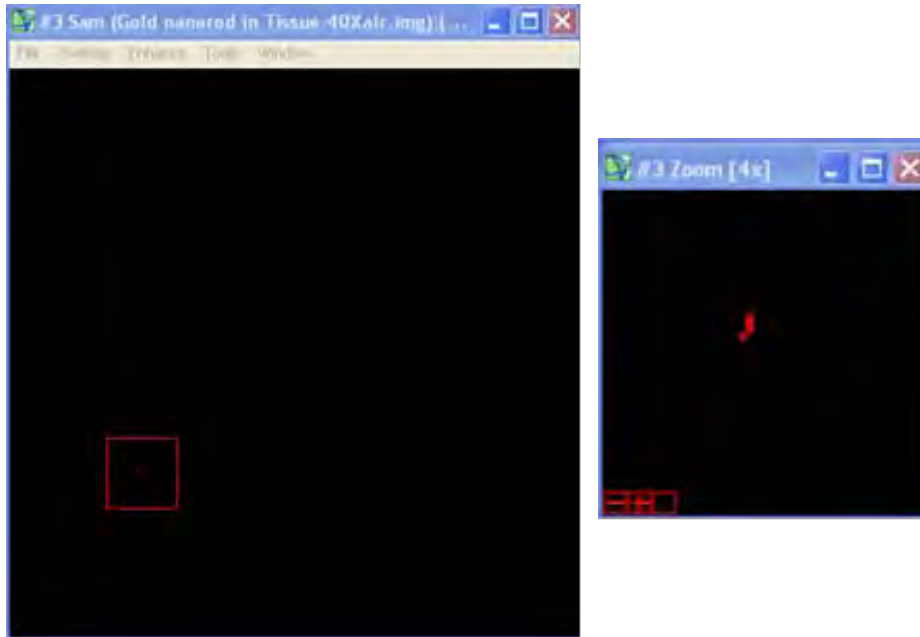


Figure 19. Classification image using a low maximum angle in SAM.

Part 6: Re-Classifying the SAM

For this example we believe other gold nanorod (GNR) particles contained in the image have spectra that are similar to the spectra collected for the first particle. Thus, we will try to add more of the particles into the classification.

1. Go back to the Spectral Angle Mapper Parameters window
2. To return to the Spectral Angle Mapper Parameters window click apply in the Endmembers Collection: SAM window
3. The Spectral Angle Mapper Parameters dialogue box opens
4. Change the Maximum Angle from 0.1 to 0.3. This time do not create a Rule image
5. Now click Preview. The new classification result is shown to the right of the dialog. You can see that more particles are classified this time
6. For Output Result, select Memory
7. For Output Rule Image, select Yes
8. For Output Result, select Memory
9. Then click OK
10. From the top of the available bands list, right click on the new image
11. Then click on "Load Band to New Display". The new classification (#2 SAM) images (Figure 22) with the original image (Figure 21) and the original classification (#1 SAM) Image at the side (Figure 20). When you compare the first and second classification images, you will see that additional particles scattered about the cell are classified as GNR when the angle was set to a greater value. The correct value for the angle must be found by experimenting with different values.

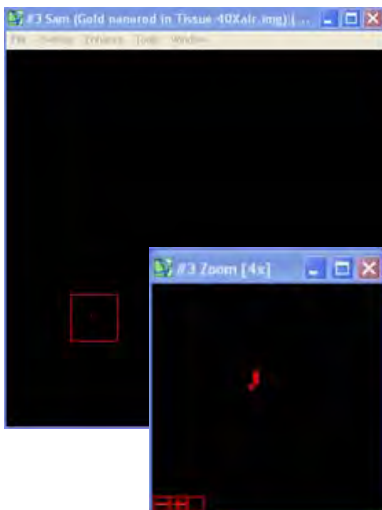


Figure 20. Original Classification (#1 SAM) image obtained using 0.1 Maximum Angle

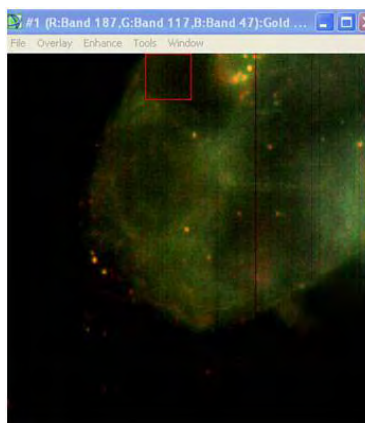


Figure 21. Original image of the GNR in tissue



Figure 22. Right New Classification (#2 SAM) image obtained using 0.3 Maximum Angle

Part 7: Linking the Displays

This feature allows you to link both the original image and the classified image to view the same areas.

1. Right click in the Original Image Window
 - a. Select Link Displays
2. Link Display dialogue box opens
 - a. No changes are needed so click OK
3. Now when you move the Zoom box in any of the three images of the original or classified image they move in sync
4. If you left click and hold in any window it will display whats in the opposite window. (see Figure 23)

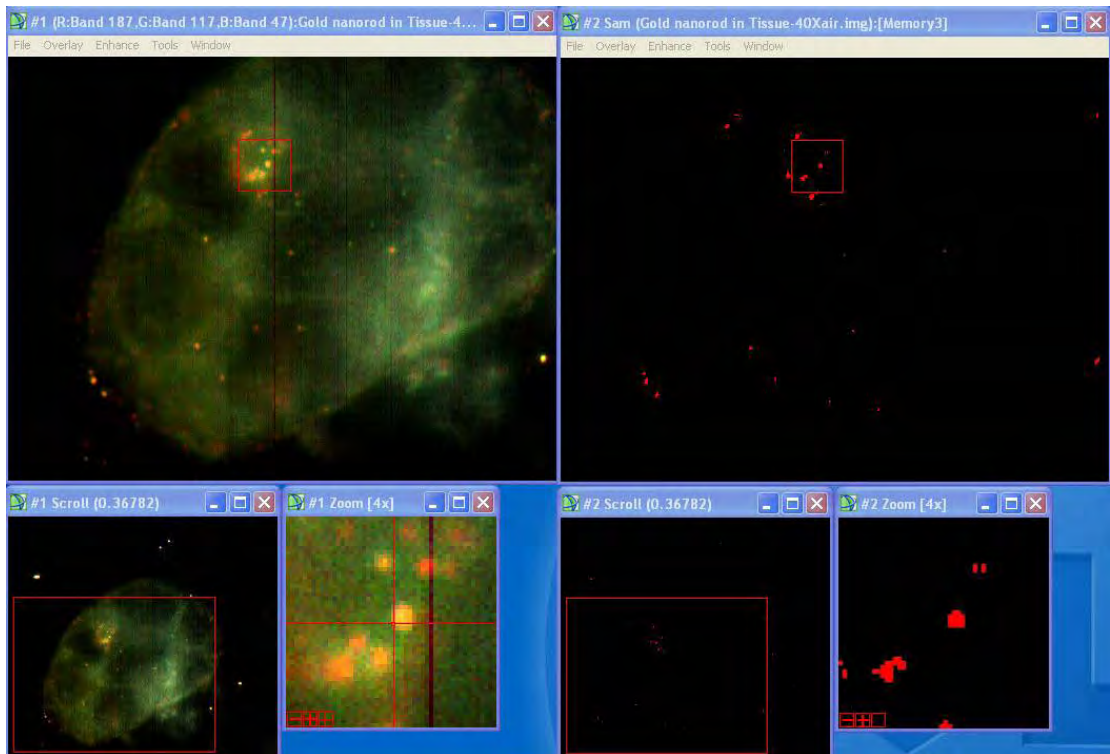


Figure 23. Linking the two displays

5. To unlink the original image and classification image right click on the original image. Then select Unlink Displays

Part 8: Overlaying the Classification Image

This feature provides you with the capability to overlay the classification image onto the original image. It will enable you to illustrate where the classification pixels are in the context of the original image.

1. In the original image window select Overlay from the menu bar
 - a. Then select Classification
2. The Interactive Class Tool Input File dialogue box opens (see Figure 24)
 - a. Select the classification file created from the SAM classifier
 - b. For this example select Memory 3 (or samples will have a filename)
 - c. You can look at the Available Bands List to help determine which one is the SAM image.
 - d. Click OK
3. The #1 Interactive Class Tool dialogue box opens
 - a. Click on the tab beside the red box marked AU-NR Tissue (see Figure 25)

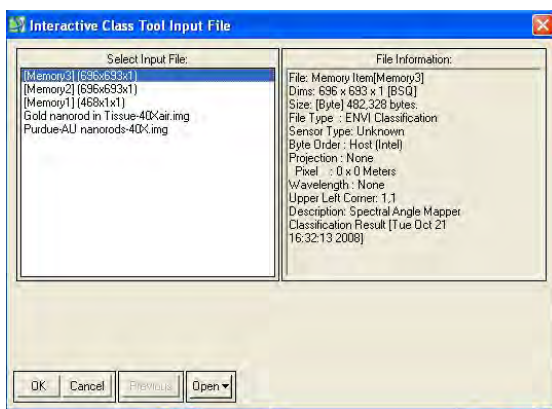


Figure 24. Selecting image to overlay

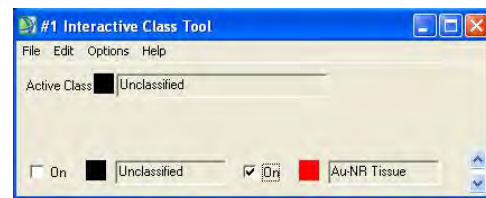


Figure 25. activating the overlay feature

4. Now you will see an overlay image of your classified picture and the original image (see Figure 26)

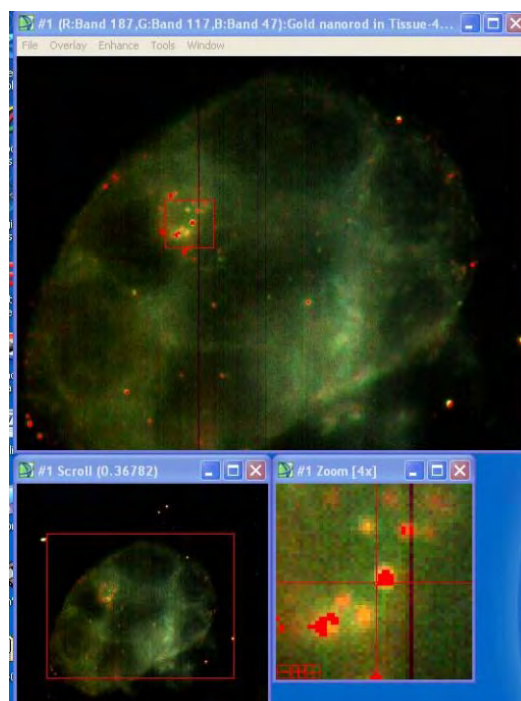


Figure 26. View of the images overlayed

Part 9: Merging Colors in a Classification Image

Sometimes multiple spectra are used to classify particles in a sample. This will give an overlay that has multiple colors. It will allow you to combine the multiple colors into a single color.

1. Open the image to be classified (see Figure 27), scroll through the Available Bands List to find the original image
2. Click on the filename to highlight it.
3. Click Display # and select New Display
4. Click Load Band to display the image
5. In the picture window (see Figure 28)
 - a. Choose Overlay
 - b. Click Classification

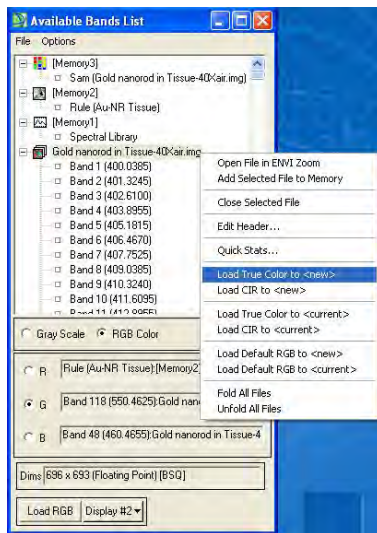


Figure 27. Available Bands List



Figure 28. Image to be given an overlay

6. The Interactive Class Tool Input File window appears (see Figure 29).
7. Select file, then click okay.

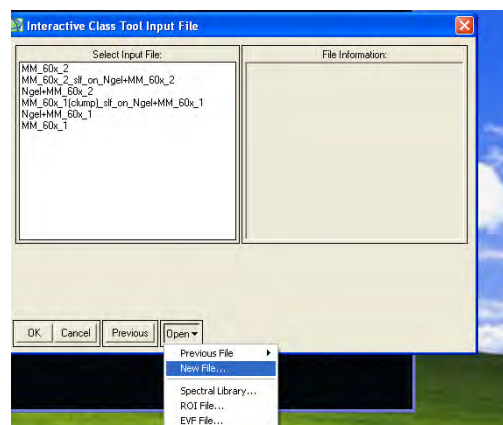


Figure 29. Interactive Class Tool Input File window

**** If the classification file is not in the Select Input File list, then click Open and select New File ****

8. An Interactive Class Tool window will appear.
9. To show the overlay
 - a. Right click on the box next to any colored square (see Figure 30)
 - b. In the Interactive Class Tool window, click Options
 - c. Select Merge classes (see Figure 31)

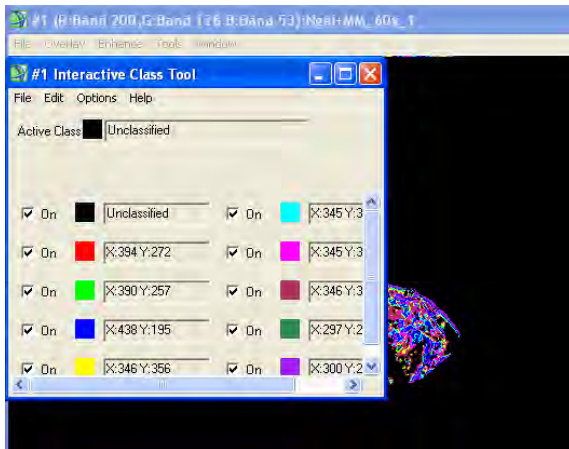


Figure 30. Overlay Image with Interactive Class Tool window

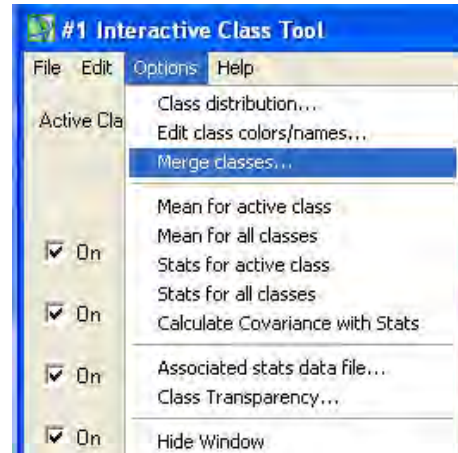


Figure 31. Options drop-down menu

10. In the Interactive Merge Classes window (see Figure 32)
 - a. Select one coordinate (X###:Y###) in the Base Class list.

****Note: The first is automatically defaulted to red****
11. In the Classes to Merge into Base list
 - a. Select all other coordinates.

****Note: Do not select the coordinate chosen in the Base Class list****
 - b. Click Okay
 - c. The classification file will automatically update to a single color (see Figure 33)

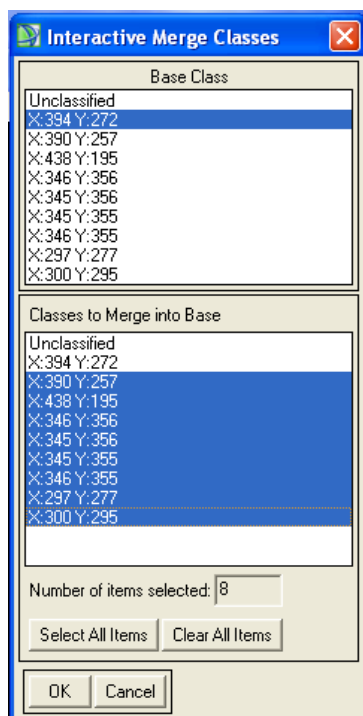


Figure 32. Interactive Merge Classes window

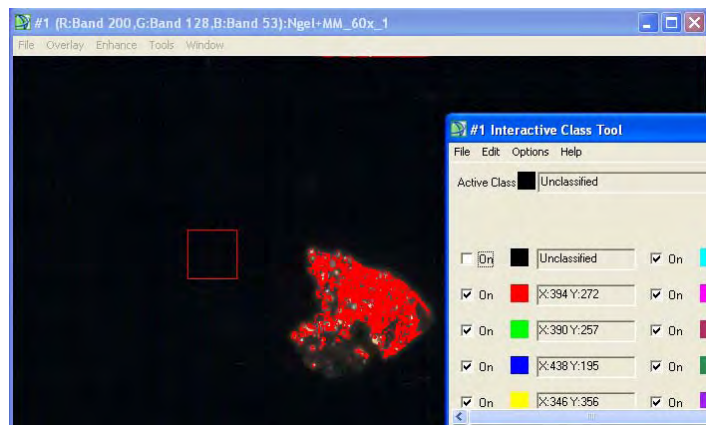


Figure 33. Classification spectra unified to a single color

Part 10: Removing Unwanted Spectra from Data Analysis

This feature provides you with the capability to remove spectra once data analysis is complete and a classification file is created.

1. In the original image window select Overlay from the menu bar
 - a. Then select Classification
 - b. Select the appropriate file
2. The Interactive Class Tool window appears (see Figure 34)
 - a. Select Options

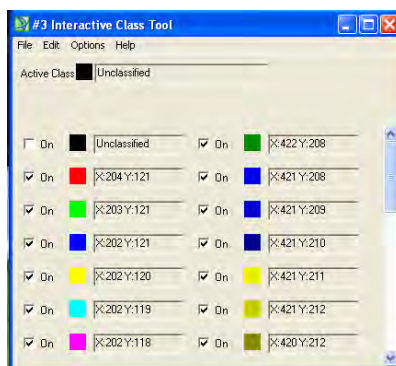


Figure 34. View of the Overlay Image

- b. Click on Class Distribution (see Figure 35).

Class Name	Npts	Pct
Unclassified	[172960]	99.802%
X:204 Y:121	[344]	0.198%
X:203 Y:121	[0]	0.000%
X:202 Y:121	[0]	0.000%
X:202 Y:120	[0]	0.000%
X:202 Y:119	[0]	0.000%
X:202 Y:118	[0]	0.000%
X:201 Y:118	[0]	0.000%
X:201 Y:119	[0]	0.000%
X:201 Y:120	[0]	0.000%
X:200 Y:120	[0]	0.000%
X:200 Y:119	[0]	0.000%
X:200 Y:118	[0]	0.000%
X:200 Y:117	[0]	0.000%
X:200 Y:116	[0]	0.000%
X:199 Y:116	[0]	0.000%
X:199 Y:117	[0]	0.000%
X:422 Y:212	[0]	0.000%
X:422 Y:211	[0]	0.000%
X:422 Y:210	[0]	0.000%
X:422 Y:209	[0]	0.000%
X:422 Y:208	[0]	0.000%
X:421 Y:208	[0]	0.000%
X:421 Y:209	[0]	0.000%
X:421 Y:210	[0]	0.000%
X:421 Y:211	[0]	0.000%
X:421 Y:212	[0]	0.000%
X:420 Y:212	[0]	0.000%
X:420 Y:211	[0]	0.000%
X:420 Y:210	[0]	0.000%
X:420 Y:209	[0]	0.000%
X:420 Y:208	[0]	0.000%
X:426 Y:125	[0]	0.000%
X:427 Y:125	[0]	0.000%
X:427 Y:124	[0]	0.000%
X:428 Y:124	[0]	0.000%
X:428 Y:123	[0]	0.000%
X:429 Y:123	[0]	0.000%
X:429 Y:122	[0]	0.000%
X:430 Y:122	[0]	0.000%
X:429 Y:121	[0]	0.000%

Figure 35. The Classification Distribution window shows the percent reactivity of each individual spectra

****You must print this window or manually record the data to be removed, as this window will minimize when you navigate away****

3. Open the spectral library (see Figure 36)
 - a. In the ENVI task bar, select File, click Open Image File
 - b. Select spectral library file to be edited. A Spectral Library Viewer window will open
 - c. Click on each spectra to create a spectral library plot
 - d. In the Spectral Library Plots window, select Options
 - e. Click on show plot key and all spectra will be listed to the right of the spectral plot

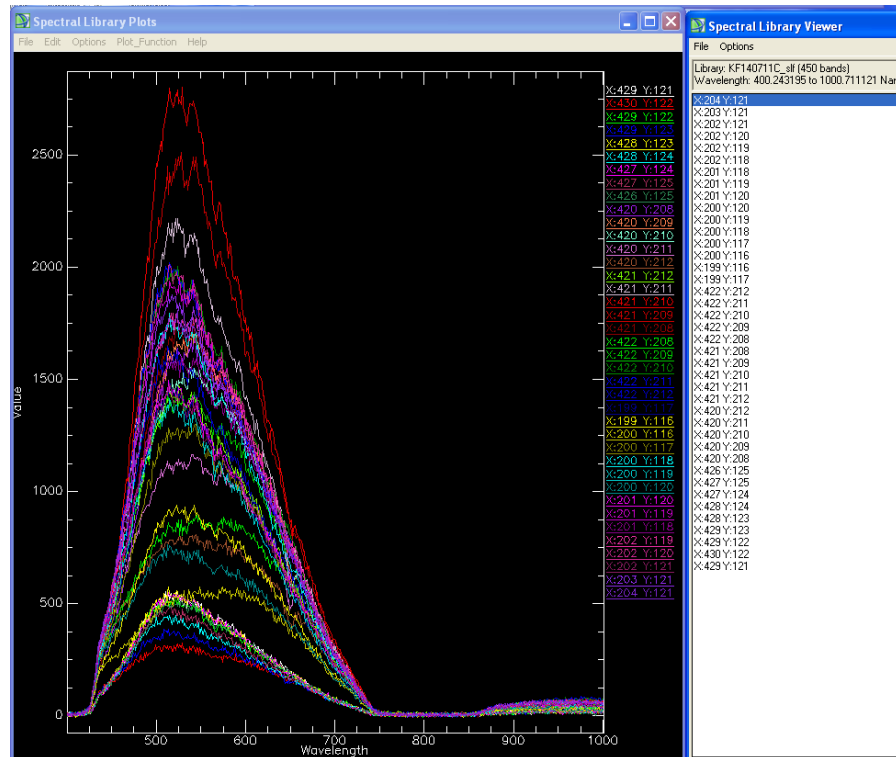


Figure 36. Spectral Library Viewer window (right) used to create the Spectral Library Plot (left)

- f. Right click on the spectra to be removed
- g. Then select Remove X: ### Y:###, for all spectra chosen (see Figure 37)

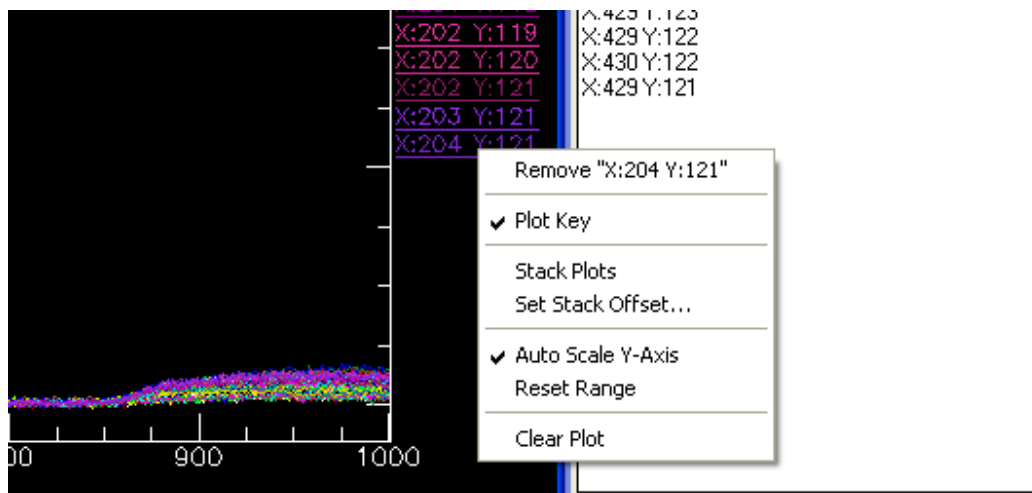


Figure 37. Selection of spectra from Plot Key to be removed

4. Save the file as a Spectral Library (see Figure 38)
 - a. Click File
 - b. Select Save Plot As
 - c. Click Spectral Library
 - d. Click Select all Items in output plots window (see Figure 39)
 - e. Then click OK
 - f. Click Choose in the Output Spectral Library window (see Figure 40)
 - g. Enter file name in Output Filename window, click Open
 - h. Click okay in Output Spectral Library window

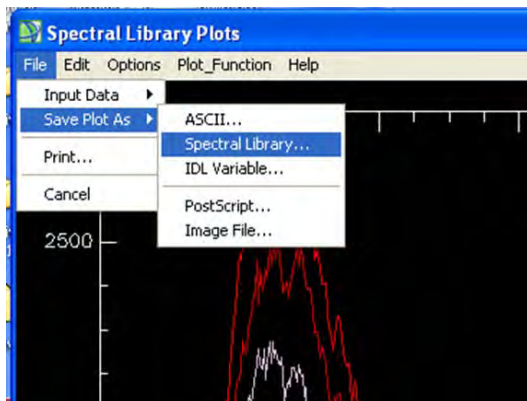


Figure 38. Saving Spectral Library



Figure 39. Output Plot to Spectral Library

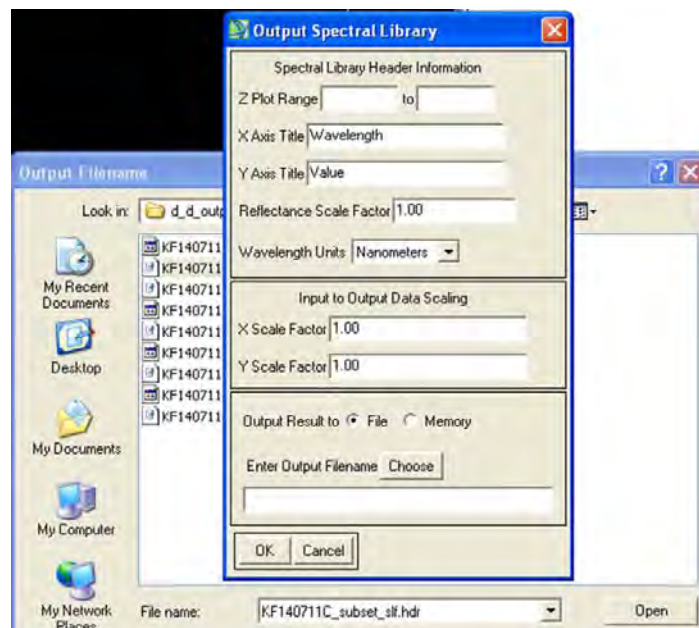


Figure 40. Saving Output Spectral Library

Part 11: Mean Spectra Analysis and Normalization

Mean Spectra Analysis and Normalization allows the user to compare spectra from separate samples. This is done by making all the intensities equal to 1.

Mean Spectra Analysis:

1. In the Image Window menu bar select Tools (see Figure 41)
 - a. Select Region of Interest
 - b. Click ROI tool

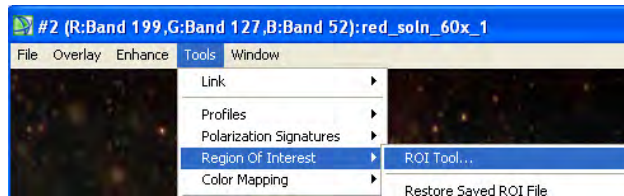
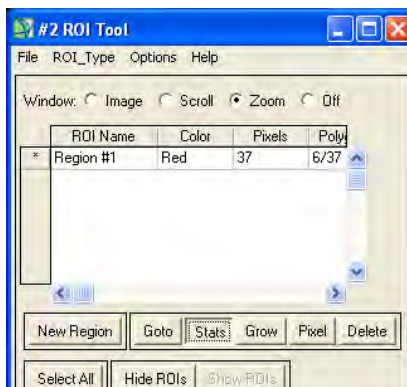


Figure 41. Opening the ROI tool

2. Highlight the Region of Interest needed (refer to Chapter 2, Part 10: Working with Regions of Interest ROIs)
 - a. Then Click the Stats radio button (see Figure 42)
3. The ROI Statistics Results window appears
 - a. Right click on the graph
 - b. Click Plot Key
 - c. Right click on the graph again
 - d. Select Options
 - e. Click New Window: Blank (see Figure 43)
 - f. A new ENVI Plot Window opens (see Figure 44)
4. To load the mean spectra into the New ENVI Plot Window
 - a. Click and drag the Mean: Region of Interest from the ROI statistics window into the new ENVI Plot Window

****Note: Multiple graphs can be loaded into this window for comparison by dragging and dropping the required data****



42. ROI tool window

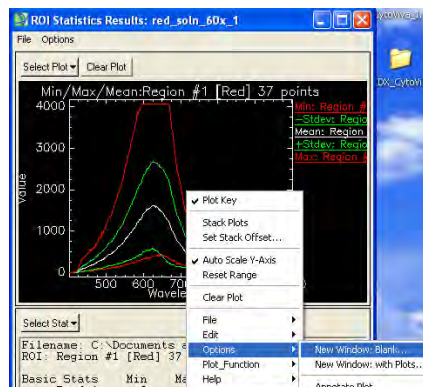


Figure 43. ROI Statistics Results

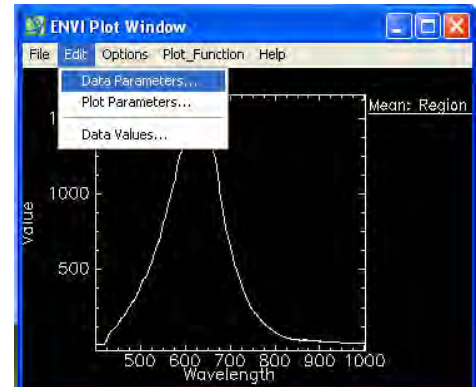


Figure 44. ENVI Plot Window

5. Click Edit from the menu bar and select Data Parameters
6. The Data Parameters Window opens. From here you can (see Figure 45)
 - a. Label the graph
 - b. Rename the selected region
 - c. Change the color of the plot: (see Figure 46)
 - i. To change the color, right click on the color square
 - ii. Move mouse over Items: ##:##
 - iii. Select a color
7. Repeat these steps to for other ROIs that you want to normalize for analysis

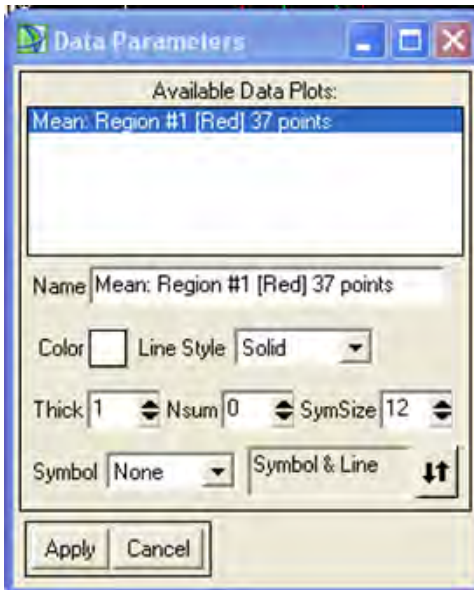


Figure 45. Data Parameters Window

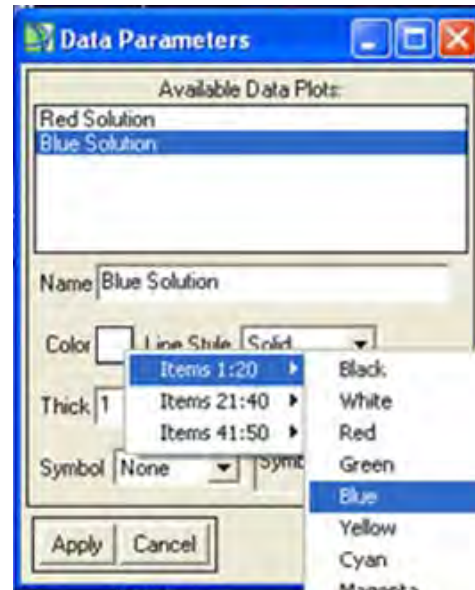


Figure 46. Changing graph color

Normalizing:

1. Using the ENVI Plot Window that has the spectra to be normalized open (see Figure 47)

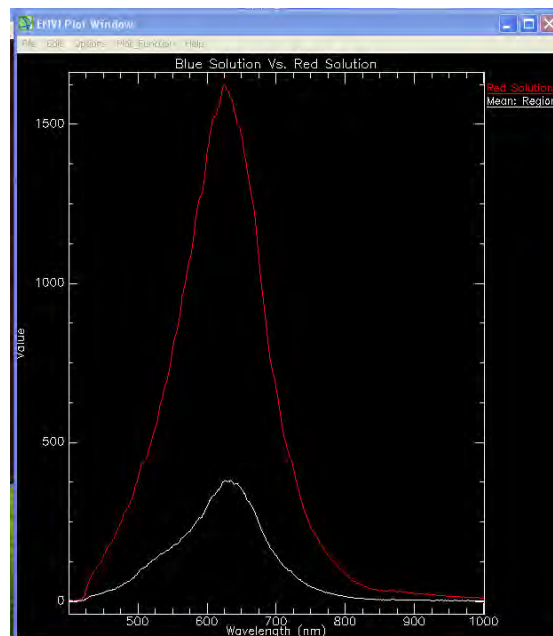


Figure 47. Spectra to be normalized

4. In the main ENVI toolbar
 - a. Select Spectral
 - b. Then select Spectral Math
 - c. The spectral math window will appear (see Figure 48)
 - d. Enter the normalizing expression: $\text{float}(s1)/\text{max}(s1)$
 - e. Click OK.
 - f. A Variables to Spectra Pairings window will appear
 - g. In the Available Spectra list, click the curve to be normalized
 - h. Click the double arrows to change Output Result to New Window
 - i. Click OK (see Figure 49)
 - j. Repeat for all desired spectra

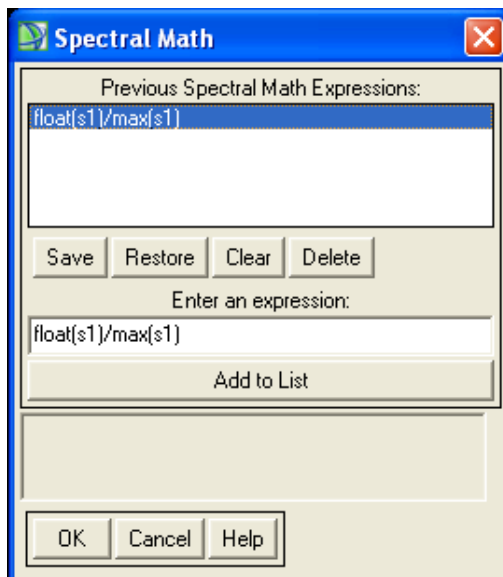


Figure 48. Normalizing expression in Spectral Math window

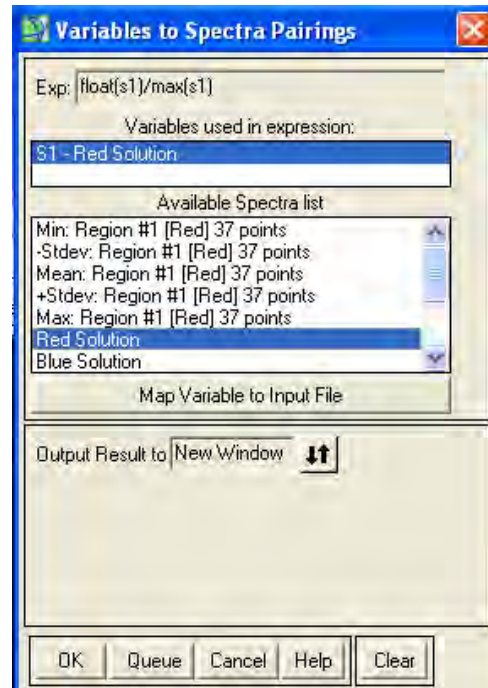


Figure 49. Variables to Spectra Pairings window

5. A new graph will appear with a vertical axis set to 1
 - a. Use Edit menu to change colors and labels (see Figure 50)

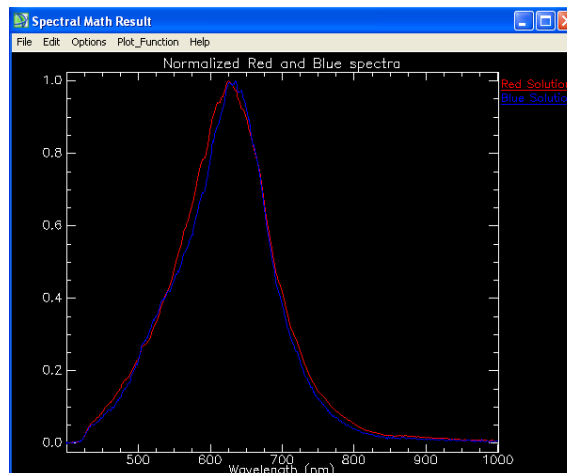


Figure 50. Normalized spectra

Part 12: Resizing an image

Resizing an image allows the user to focus on an area of interest and cut extraneous parts of the image.

1. In the ENVI toolbar
 - a. Select Basic Tools
 - b. Click Resize Data (Spatial/Spectral) (see Figure 51)
 - c. The Resize Data Input File box Opens (see Figure 52)
 - d. Select file to resize
 - e. Click Spatial Subset radio button
 - f. The Select Spatial Subset window appears
 - g. Click the Image radio button



Figure 51. Opening the Resize window

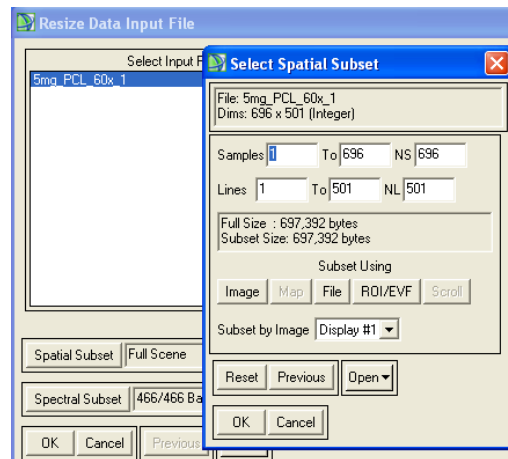


Figure 52. Select Spatial Subset window

2. A Subset by Image window appears. The whole scan is shown in this window. There is also a red box that appears (see Figure 53)
3. Click and drag the corners of the red box to the preferred size
 - a. Click OK to exit Subset window
 - b. Click OK to exit Spatial Subset window
 - c. Click OK to exit Resize Data window
 - d. Save file accordingly and the newly resized image will appear.

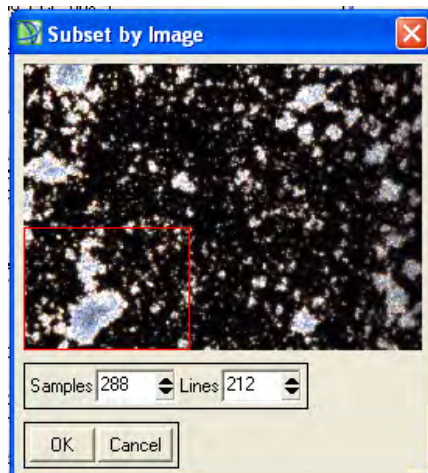


Figure 53. Subset by Image window

Part 13: Spectral sub-setting an image

Spectrally sub-setting an image allows the user to focus on specific wavelengths of light. This is useful in removing second order harmonics and other known reflective contaminants.

1. In the main ENVI toolbar
 - a. Select Basic Tools
 - b. Click Resize Data (Spatial/Spectral) (see Figure 54)
 - c. Choose image to resize in the Select Input File list
 - d. Click the Spectral Subset radio button (see Figure 55)

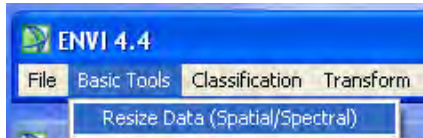


Figure 54. Opening the Resize window

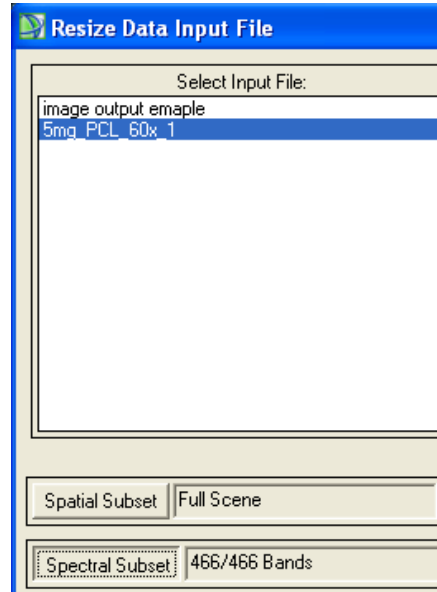


Figure 55. Resize Data Input File window

2. The File Spectral Subset window appears
3. Select the range of spectra to become the subset by clicking and scrolling (see Figure 56)
****To select non-contiguous values hit the Ctrl key****
4. Click OK to exit Spectral Subset window
 - a. Click OK to exit Resize Data window
 - b. Save file accordingly and the newly sub-setted image will appear.

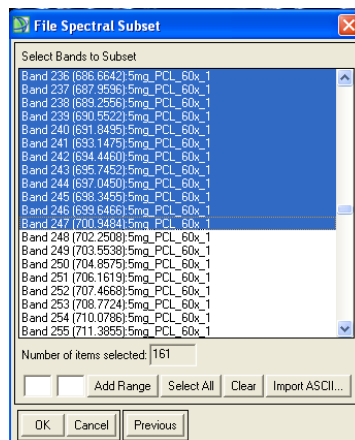


Figure 56. File Spectral Subset window

Chapter 6: Quantitative Spectral Analysis

In a hyperspectral scan, the spectral features are produced by specific light scattering and absorption properties of the specimen materials that are recorded at each image pixel. Light scattering is more strongly influenced by surface chemistry, whereas light absorption will depend on internal as well as surface properties of the specimen. The recorded spectrum also contains features that are properties of the light source that are used to illuminate the specimen, and also of optical filters that alter the light reaching the specimen. The recorded data actually shows the spectrum of the light from the source, going to the sample where it is modified by specific spectral features from the specimen. So, for example, light absorption by a specimen over certain wavelengths may reduce the recorded light intensity at those wavelengths, but leave what is essentially a spectrum of the light source at the other wavelengths. This occurs when the sample contains light absorbers such as dyes and pigments, like melanin, that are intrinsic to the sample, or if there are dyes that are added to create contrast from sample structures. For example, the hematoxylin and eosin stains will modify the spectrum differently by virtue of their distinct light absorption properties. This situation is common for all of the microscope methods that directly sample the illumination, such as bright field, reflected bright field and dark field modes.

The situation is different for spectra of nanoparticles made from the noble metals. For these, recorded spectra show light scattered within narrow peaks that are caused by plasmon resonances, where nanoparticle electrons resonate at frequencies that are influenced by the dielectric materials properties surrounding the particle. When recorded with the dark field method, the recordings are exclusively from the scattered light and do not contain any of the light absorption features. So, instead of recording the light source spectrum after it has been modified by features of the specimen, the recording contains discrete resonance peaks whose strengths are set by intensity of the light source at the resonance wavelengths. Of course if the specimen contains dyes, pigments and nanoparticles, both kinds of effects can occur together in the recorded spectrum.

For all types of HSI spectra simple procedures can be used to improve accuracy and repeatability, so that results can more easily be compared between recordings. One of the simplest methods is "spectral normalization". This procedure is able to counter the effects of the camera and spectrograph on the spectral recording. Both of these components act to reduce the strength of the recorded spectrum at longer wavelengths. The idea of spectral normalization is to compensate for these effects so that spectral characteristics of the sample are clearly seen in the data. To perform this, it is first necessary to have the light source spectrum that was obtained with your HSI system.

Part 1: Recording of the Light Source

The lamp spectrum has already been recorded on each HSI microscope and provided as a spectral library called "Halogen Lamp Spectrum". If you have an older system you can obtain the lamp spectrum with the following procedure.

1. If you are using a CytoViva condenser
 - a. Remove the liquid light guide from the input port by unloosening the screw and pulling out the liquid light guide.
2. Set up a scan
 - a. Set the exposure time to 0.005 seconds and the number of lines to 20. Keep all other settings the same
 - b. Enter a file name like Light Source and start a preview window
3. Setting the light source up
 - a. Move the lens turret to an empty position
 - b. Aim the end of the liquid light guide into the empty position and watch for the light to register on the preview window
 - c. You will want to tape the guide in place while you run the scan

****Make sure that the values in the Preview data are under 4000****

4. Start the scan. After finishing, set a rectangle ROI in the main image window
5. Select Stats in the ROI tool. The spectrum, along with the maximum, minimum and standard deviation spectra will be displayed
6. Right click in the plot window
7. Select Plot Key
8. Right click again and select Options
9. Select New window: Blank
10. Left click on the legend for the white curve that contains the mean spectrum
11. Drag the name to the blank plot. It will show the lamp spectrum by itself
12. Save the plot as a spectral library so you can do further work with the lamp spectrum (see Figure 57)

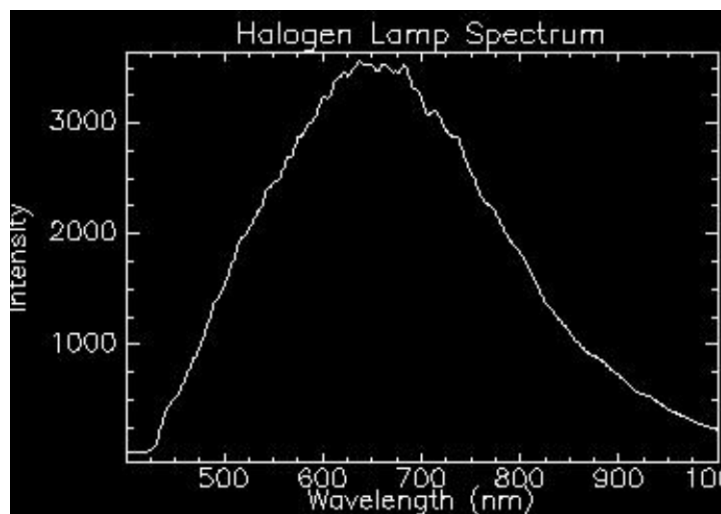


Figure 57. The spectrum of the halogen lamp acquired with a Pixelfly camera and spectrograph on CytoViva HSI. The spectrum shows the shape of the halogen lamp spectrum

****Note: This contains effects of the camera and spectrograph upon the actual halogen light spectrum emitted from the light source****

Part 2: Spectral Normalization of the Lamp

This procedure removes the effect of the lamp spectrum so that sample features are seen more clearly.

1. First set the value of the highest point on the lamp spectrum to unity. If you recorded a new lamp spectrum, go to Basic Tools located on the main ENVI menu, select Spectral Math

****The halogen lamp spectral library provided by CytoViva has already been adjusted this way****

2. Type in the expression: $\text{float}(S1)/\text{max}(S1)$ and add it to the list of expressions. Select the expression and hit OK
3. Use the Save button to save this expression to a special folder where you will keep expressions. You can use Restore to bring the expression back for another session
4. Assign the lamp spectrum to the variable S1. If the lamp spectrum is already open in a plot window or saved as a spectral library, you will see the spectrum in the list. Click on the name to select it
5. Choose to save the results in a new plot. Click OK
6. The normalized lamp spectrum is plotted (see Figure 58)

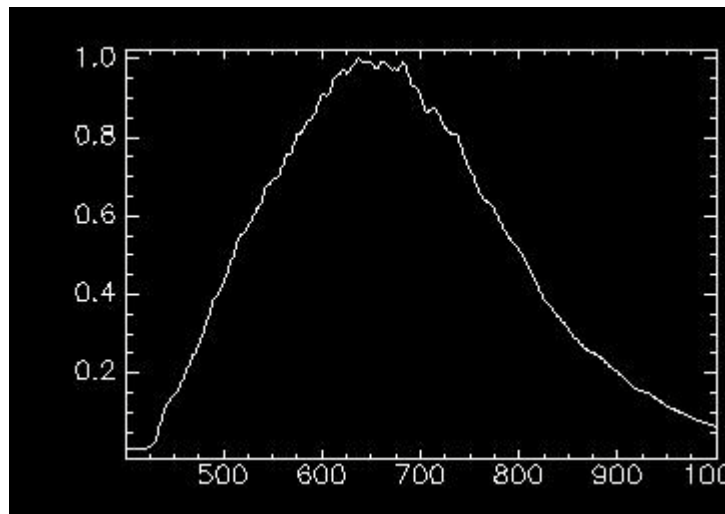


Figure 58. Normalized lamp spectrum

Part 3: Spectral Normalization of the Specimen

Now you have a lamp spectrum for your HSI system. Next, we will use it to correct your specimen spectra for instrumental effects. The correction is to divide the specimen spectrum by the normalized lamp spectrum. There are different ways to do this. You can correct a single spectrum from a Z profile, or correct a mean spectrum from an ROI. Alternately, you can correct all of the spectra in a data cube.

1. Enter or Restore the correction expression: $\text{float}(S1)/\text{float}(S2)$. Add it to the list of expressions and save it to use later. Select the expression and click OK
2. Select S1 and make one of the following assignments
 - a. To correct a single specimen spectrum, select the curve you want from the list. It will appear there if it is also in a plot window
 - b. To correct a data cube, use the file assignment option to browse for the specimen's HSI data file. If you do this, the entire image will be corrected
3. Choose a new window to plot the corrected spectrum. A new filename for the corrected data cube can be chosen
4. In the assignment window, select the normalized lamp spectrum for S2 (see Figure 59 and 60).

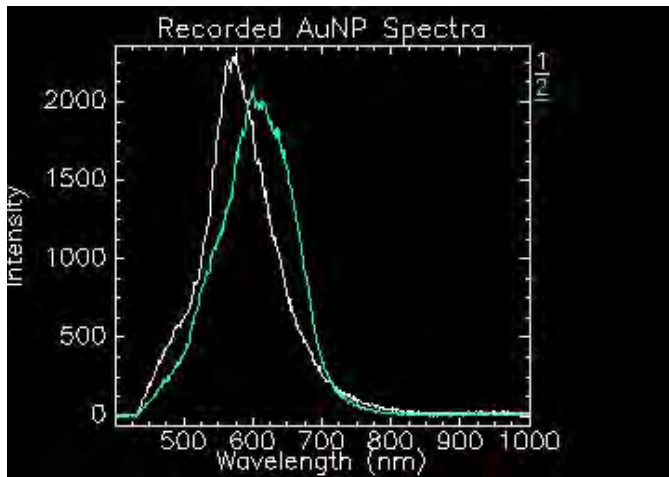


Figure 59. The uncorrected spectra of nanoparticles

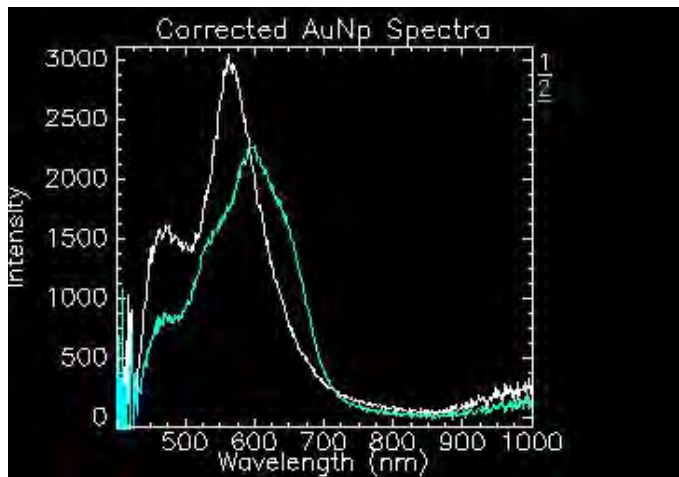


Figure 60. After correction for instrumental two effects

****Note: The correction results in noise where the uncorrected spectrum had no intensity. This noise is not related to features of the particles****

Part 4: Correction for second order diffraction

The range of the spectrograph used with CytoViva is from 400 nm to 1000 nm. Since the spectrograph is a grating type, the spectrum starting at 400 nm also starts again at 800 nm because of the second order diffraction of the grating. This means that there are spectral values between 800 nm and 1000 nm that "mirror" the values from 400 nm to 500 nm. Values between 400 nm and 800 nm are always obtained from the fundamental wavelengths, i.e. the first order diffraction pattern, and are never contaminated by the second order.

To obtain true values out to 1000 nm, we recommend using a 500 nm long pass filter to block the light between 400 nm and 500 nm which will produce interference past 800 nm. This gives two free spectral ranges:

- ① 400 nm - 800 nm without the filter
- ② 500 nm to 1000 nm with the filter

Since the long pass filter does not transmit all of the light past 500 nm, it is necessary to also record the light source spectrum when using the filter to do spectral normalization.

Part 5: Conversions to Reflectance and Absorbance

The light absorbance properties of materials can be determined from the absorption spectrum that is recorded as light passes through the material. Using HSI, the absorption of materials on glass slides can be found and classification of the material based on the absorption property can be carried out.

Transmitted Light:

To obtain the absorption spectrum of a specimen on transparent surface, use a bright field illumination which sends light directly through the glass slide into the microscope objective.

1. Locate the area of interest on the specimen and center it in the scan area
2. Draw an ROI around the region
3. Save the mean spectrum in a plot
4. After recording the specimen, replace it with a blank slide, or a region of the same slide that does not contain the specimen
5. Open the ROI tool and use the same region to obtain the mean spectrum of the region
6. Save this to a plot
7. Calculate the absorption spectrum using the spectral math expression:
 - a. $\text{alog(float}(S1)/S2)$. Assign S1 to the spectrum of the blank and S2 to the spectrum of the specimen. Choose a new window to create the absorption spectrum. The light absorption is maximum at the peak of this spectrum, where also the light reflectance is minimum

Reflected Light:

To obtain absorption spectrum of an opaque sample/material, use a reflected light illumination which sends light that bounces off the sample back into the microscope objective.

1. Locate the area of interest on the specimen and center it in the scan area
2. Draw an ROI around the region
3. Save the mean spectrum in a plot
4. After recording the specimen, replace it with a reflectance target. A suitable target is made by LabSphere (model SDM-100-DU or SDM-050-DU)
5. Open the ROI tool and use the same region to obtain the mean spectrum of the region
6. Save this to a plot
7. Calculate the percent reflectance spectrum using the spectral math expression:
 - a. $100*\text{float}(S1)/S2$. Assign S1 to the spectrum of the specimen and S2 to the target spectrum. Choose a new window to create the absorption spectrum. The light reflectance is maximum at the peak of this spectrum, where also the light absorption is minimum

Chapter 7: Clean-up and Supplies

To properly take care of the CytoViva Hyperspectral system there are a few steps that are necessary to do when you are done using the system to keep the system performing at its top level.

Part 1: Daily Clean-Up of the System

When you are done using the CytoViva Hyperspectral Imaging System it is important to clean the excess immersion oil off the condenser and the objectives. Also turning off components of the system are necessary as well.

1. Use a KimWipe or lens paper to wipe off any excess oil from the CytoViva condenser and the objectives
2. Power off the light source
3. Turn the Dage camera off by unplugging the power cord connected directly to the top of the camera
4. Optional – turn the stage controller unit off using the switch on the back of the controller box.
5. Cover the microscope with the CytoViva microscope cover or similar
6. Use cleaning solution about every 2-3 months to clean the CytoViva condenser and the objectives.

****Letting the immersion oil sit and dry on the condenser and the objectives can cause a “gum-like” film which can cause damage to the lens****

Part 2: CytoViva Supplies

Below listed are supplies that are recommended when using the CytoViva system for imaging and clean-up.

Fisher Scientific:

Pre-cleaned slides: 1 x 3mm plain glass slide, Part #: 12-544-1

Pre-cleaned coverslips: .17mm thickness, No. 1 slides (two sizes below)

18mm x 18mm: Part #: 12-542A

55mm x 24mm: Part #: 12-544-18

Cargille Immersion Oil: Type A, 16 fl. oz., Part #: 12-368B

TexWipes: good wipes for cleaning, Part # 18-315A

Fisher Brand Optical Lens Cleaner: use for cleaning, Part #: 22-143-974

Schott North America Inc.:

Contact: Lynn Larcheveque, lynn.larcheveque@us.schott.com, (502) 657-4412

****Mention that CytoViva referred you****

Ultrasonically cleaned slides and coverslips: **recommend for best imaging results**

SLIDES: Part Number: 1055132, Nexterion Glass B (Ultrasonically clnd)

COVER SLIPS: Part Number: 1098576, Nexterion Glass D type#1 D263 ****Custom Coverslips****

Glossary

Image File:  This is the file of the original image scanned with the spectrophotometer.

Spectral Library File:  This file contains the different spectra that the user will collect from the scanned image.

Rule Image File:  This file contains the Rule image of the sample.

Classification (SAM) File:  This contains the classification (SAM) Image of the spectra that matched the endmembers.

Rule Image: The rule image is the displayed grayscale image showing the degree of matching between the known and unknown spectra from each image pixel. The Rule image is shown on a scale of light to dark the relative degree to which unknown spectra in each image pixel match the known spectra.

Spectral Angle Mapper Classification (SAM): an automated procedure for determining if a known material is present in the input image, and the pixels containing the material. SAM accomplishes these tasks by comparing unknown spectra in Hyperspectral imagery with known laboratory spectra for the material in question.

Classification (SAM) Image: this image shows the location of pixels whose spectra match that of the endmember.

Header File: A separate file created with the Hyperspectral image file, with the same name. This file must be copied with the image file when data is transferred. The header file includes information for CytoViva-ENVI to allow the image program to be opened and used.

Endmember: This is any spectra that have been chosen as important for identification purposes in a sample.

Output File: This is a file that you create to include several different output files such as: Spectral Library Files, Header Files, Spectral Angle Mapper Files, etc. This file will allow you to easily access this information when needed.

Z Profile Average Window: Setting the size windows will either decrease or increase the noise in the plotted spectrum.

Plot Key: This is the key that contains the labels of your spectral curves chosen.

X Axis (Default): The X-Axis default is the Wavelength. By moving the cursor over the point of the peak you can see the wavelength of the spectra in the selected location.

Y Axis (Default): The Y-Axis is the Value. This provides the intensity levels of the spectra.

Data Parameters: this can be found in the plot key by click on the image menu bar and selecting edit, then select Data Parameters. The Data Parameters will allow you to change the Key Plot names, line style (dotted, thickness) and color.

Plot Parameters: this can be found in the plot key by click on the image menu bar and selecting edit, then select Plot Parameters. The Plot Parameters will allow you to change the title, X & Y axis, background and foreground colors, and the font.

Spatial Resolution: The minimum spatial separation that can be detected in the image. The control of spatial and spectral resolutions work the same way.

Spectral Resolution: The minimum separation in wavelengths that can be detected. The low spectral resolution setting is equal to half that of the Hi setting. Therefore if the hi resolution were 1.5 nm, the low resolution would be 3 nm.

Trouble Shooting

1. What is the number for Technical Support?

1-888-737-3130

2. After the scan has finished the Image on the screen is Black?

One of the slide bar wasn't opened or closed at the proper time. Refer to the Manual, Chapter 2: Quick Start Guide, Part 3: Starting the Scan, numbers 6 & 7.

3. The scanned image is out of focus?

Before you scan your sample be sure to double check that the sample is in focus. Sometimes the slightest movement can make the sample move.

4. When I rescan an image the original image is still showing?

Go to the Available Bands list and choose close all files and then in the Available Bands list and open the new image you just scanned. You can refer to the Manual in Chapter 2: Quick Start Guide, Step 3: Starting the Scan, and number 8 which provides more detail.

5. There is a black area at the right or left end of my scanned image?

This is due to the slit not being pushed in all the way on the spectrophotometer. Call Technical Support.

6. I can't get spectra past 750nm?

CytoViva offers a different bulb for the Dolan Jenner than the dichroic bulb provided. The bulb is an aluminum bulb that will provide light output to about 950 nm. Please call CytoViva, Inc. to order the bulb at 888-737-3130.